Biological Role of Type IV secretion systems in *Burkholderia cenocepacia*

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ABSTRACT

Burkholderia cenocepacia is an opportunistic pathogen in patients with cystic fibrosis, causing infections with difficult treatment due to its high virulence and its multiple antibiotic resistance. Over the years, various mechanisms have been described which help to understand how *B. cenocepacia* establishes an appropriate infection environment. Bacterial secretion systems, machineries allowing effector delivery to eukaryotic host cells and DNA exchange, are highly related to pathogenesis. In *B. cenocepacia*, various secretion systems have been described, grouped into different types according to their structure and function: one Type II, one Type III, two Type IV, and one Type VI Secretion System. There are contradictory reports about their biological role and contribution to virulence. *B. cenocepacia* presents two Type IV Secretion Systems, one encoded on chromosome II (VirB/D4 T4SS) and the other encoded in a plasmid (Ptw pT4SS). Previous work has suggested a DNA transfer function for VirB/D4 T4SS. pT4SS was named Ptw according to its involvement in the Plant tissue watersoaking phenotype. Moreover, it was proposed to translocate effectors to the host and to contribute to intracellular survival. However, other experiments have shown that there is no difference in intracellular bacterial survival in strains with pT4SS mutations. In addition, previous assays carried out in our laboratory, have demonstrated a DNA transfer function for *ptw* genes. Therefore, in the present work we establish an analytical approach in order to study the biological role of T4SSs of *B. cenocepacia*. Strains with deletions in various secretion systems were assessed in onion watersoaking assays and bacterial killing assays in order to compare the corresponding phenotypes. In contrast to what was previously reported, no significant differences were found in ΔpT4SS mutant and wild type strain, both of them causing watersoaking phenotype. This clear-cut result confirms that the pT4SS does not contribute to the watersoaking phenotype. Interestingly, in bacterial killing assays, an increase in survival of the prey (*E. coli*) was observed in ΔT4SS mutants compared with wild type strains. This phenotype was also observed in ΔT6SS mutants, suggesting that both secretion systems are coordinated for bacterial killing. Experiments addressing the expression of both sets of genes in the mutant strains, as well as experiments to test intracellular infections and DNA transfer to eukaryotic cells, could not be performed due to the lockdown; the proposed experiments are detailed, and expected outcomes are discussed.

Index

Abbreviations

Ap	Ampicillin
Bcc	*Burkholderia cepacia* complex
bp	Base pairs
CF	Cystic fibrosis
CFU	Colony forming unit
Cm	Chloramphenicol
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
Dpi	Days post infection
eGFP	Enhanced green fluorescent protein
FBS	Fetal bovine serum
Gm	Gentamycin
HGT	Horizontal gene transfer
IPTG	Isopropyl β-d-1-thiogalactopyranoside
Kb	Kilo base pairs
Km	Kanamycin monosulphate
Mb	Mega base pairs
MOI	Multiplicity of infection
Nx	Nalidixic acid
OD_{600}	Optical density at 600 nm
oriT	Origin of transfer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Ptw	Plant tissue watersoaking
Sm	Streptomycin
spp	species
SS	Secretion systems
T2SS	Type II secretion system
T3SS	Type III secretion system

T4SS Type IV secretion system

T6SS Type VI secretion system

Tp Trimethoprim

X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

1. Introduction

1.1. Bacterial interactions in the environment

In nature, it is common to find interactions between different organisms, which operate in a certain environment, responding appropriately to stimuli in order to adapt and survive. In this diverse environment, bacteria have evolved a complex set of interactions with the milieu and with other coexisting organisms, both prokaryotic and eukaryotic, whether it is to cooperate or to compete with them, in order to gain optimal access to resources.

1.1.1 Bacteria – Bacteria interactions

The formation of microbial communities that coexist with each other has been observed, forming complex biological systems where phenomena that allow them to share characteristics may occur, increasing their ability to adapt, or in counterpart, developing a biochemical battle that would annihilate a competitor species (Mitri & Richard Foster, 2013). Microorganisms compete for nutrients and space. This competition can be indirect, for instance, through the consumption, leaving its competitor with fewer resources to develop; or direct, through biochemical weapons, such as toxins (Ghoul & Mitri, 2016). However, bacterial cooperation is also observed when, for example, certain microorganisms share genetic information that allows them to encode resistance against a given antibiotic (Chellat et al., 2016).

Due to the increasing development of bioinformatics, sequencing and structure revealing technologies, it has been possible to characterize various molecular mechanisms through which interactions occur in microbial communities. These interactions are regulated by the environment, in which microorganisms are found. For instance, many expressed phenotypes are regulated by a microbial mechanism called *Quorum Sensing*. This system would allow, through diffusible molecules named autoinductors, to evaluate the local population density and monitor the immediate environment response. In this way, a conditioned behavior against an ecological stimulus, whether attack or cooperation, would be possible (Nadell et al., 2016).

Sharing of genetic information among bacteria is a frequent phenomenon, which may be accomplished through a number of horizontal gene transfer mechanisms (HGT), such as transformation, transduction and conjugation (T. G. Villa et al., 2019). In fact, conjugation is an important mechanism that allows microorganisms to adapt rapidly to environmental changes, such as the massive human introduction of antibiotics in the last century.

Bacterial Conjugation consists on DNA transfer unidirectionally from a donor cell to a recipient cell (Waksman, 2019). DNA transfer requires a complete machinery that it is encoded by a conjugative plasmid or integrated conjugative elements. Plasmids are common in bacteria, and are a collection of genetic information storage in circular autonomous DNA molecules. In order to accomplish DNA transfer by conjugation, various elements must be present: an origin of transfer (*oriT*), the short DNA sequence where the process starts and ends, which must be present in the DNA molecule to be transferred; and a group of proteins that have specific functions, including DNA processing reactions, and the active transport in the recipient cell (Llosa & de la Cruz, 2005).

Conjugation starts with the relaxosome assembly. The relaxosome is the nucleoprotein complex where conjugation will be started, and includes the *oriT*, and a key protein called relaxase. This protein plays an important role: first, it catalyses a nicking reaction on a single strand of *oriT* (*nic* site) and later, at the cytoplasmic membrane, it binds the secretion system through interactions with the transport machinery (Waksman, 2019). These bacterial secretion systems will be covered in more depth in section 1.2.

Sharing antibiotic resistance is but one example of the advantageous traits that can be spread among bacterial populations through conjugation. In fact, there is a relationship between mobile elements and genes that allow adaptation to a particular environment, such as hydrocarbon degradation, nodulation, tumorigenesis, and pathogenesis (Funnell & Phillips, 2004).

Bacteria also compete against each other to utilize resources in order to survive. Specialized biomachines have been design to accomplish this purpose, such as Type VI secretion systems (T6SS), that have been related to bacterial killing (Villa et al., 2019). These systems will be cover in more depth in the section 1.2.2

1.1.2 Bacteria – Eukarya interactions

Interaction between prokaryotic and eukaryotic kingdoms have been fully documented through history using ecological and bioinformatic approaches. Symbiotic relationships between organism have been described such as mutualism, for instance in gut microbiota in animals, or parasitism in pathogen human infections (Kado, 2009; Schluter & Foster, 2012). In particular, human bacterial pathogens have been studied deeply, due to their clinical relevance in infection disease (Casadevall & Pirofski, 1999).

Interactions between pathogens and hosts are dynamic relationships, since pathogen virulence and host defense mechanisms may be conditioned by genetic factors and environmental elements (Pirofski & Casadevall, 2008). Virulence, defined as a capacity of certain microorganism to provoke infections, may be influenced by different factors, such as the number of infecting bacteria, the bacteria virulence factors, the route of entry into the body and nonspecific host defense mechanism. This may cause that some microorganisms are more aggressive than others. Generally, for intracellular bacteria, that survive inside the eukaryotic cell, the host owns molecular mechanisms in order to eliminate them. However, some microorganisms possess mechanisms to protect themselves from the host defense, such as avoiding the effect of these lysosomal enzymes or preventing the phagosome-lysosome fusion (Casadevall & Pirofski, 1999).

During the infection process, bacterial pathogens use different molecules called virulence factors to promote their survival. These virulence factors usually represent an energy consumption, therefore they are commonly regulated and expressed during the infection process. In order to accomplish their objective, virulence factors are secreted to the extracellular milieu or to the host cell cytoplasm throw secretion channels called bacterial secretion systems (Büttner & Bonas, 2003).

1.2. Bacterial Secretion Systems

Bacterial secretion systems (SS) comprise a wide range of nanomachines made up of multiprotein complexes situated across the bacterial membrane/s, allowing molecular exchange between the cell and its environment. Substrates of different nature may be transported by these systems, such as enzymes, toxins, DNA and DNA-protein complexes, which may have a direct influence on bacterial pathogenesis (Costa et al., 2015). There are different kinds of bacterial secretion systems. Their design will differ depending on their function and the location where they perform this function. In Gram-negative bacteria, secretion requires specialized machinery due to the challenge of crossing two, and in some cases, up to three membranes. In fact, some secreted proteins are transported in two separate steps, with the intervention of two different secretion systems (Green & Mecsas, 2016).

Dedicated secretory systems in Gram-negative bacteria are grouped in families numbered Type I to Type IX, except for Type VII, only present in Gram-positive bacteria (Pena et al., 2019). Each system carries a specific subset of proteins. Types III, IV and VI SS (T3SS, T4SS and T6SS) (Figure 1.1) stand out for their ability to inject their substrates

into an eukaryotic target cell, and their involvement in virulence and pathogenicity (Zalguizuri et al., 2019). Both T4SS and T6SS interact with different target cells, including bacteria and eukaryotic cells, through their secreted substances. (Russell et al., 2014a), while T3SS target only eukaryotic cells (dos Santos et al., 2020).

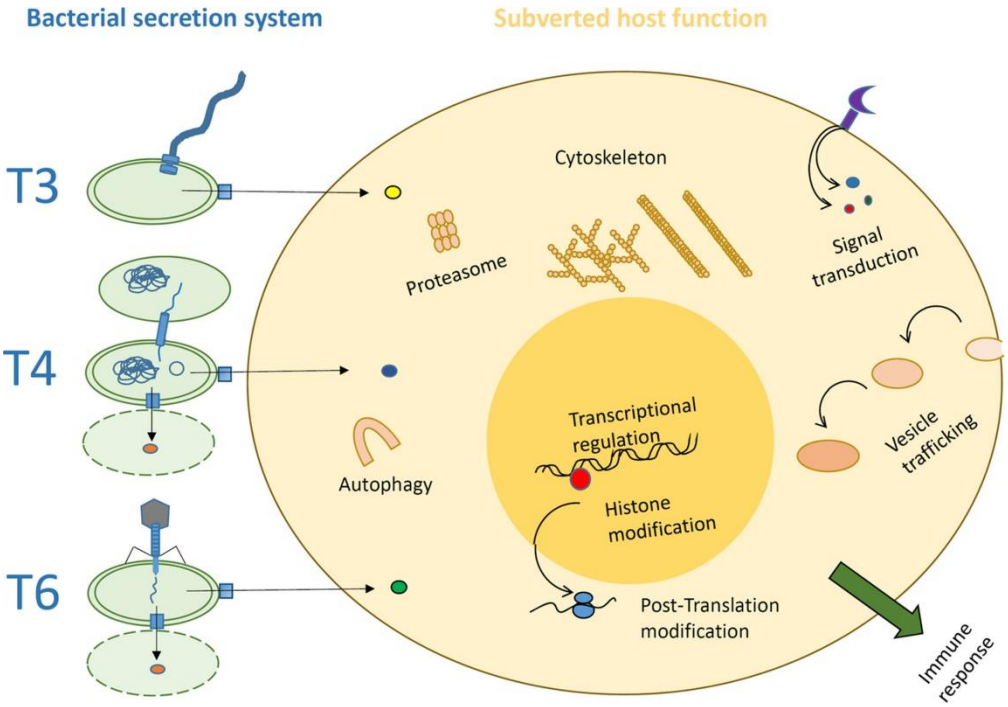

Fig 1.1 Schematic representation of T3SS, T4SS, and T6SS and their biological roles. Bacteria (in green) are depicted with each SS and their evolutionary original role: flagella (T3SS), DNA transfer (T4SS), and phage tails (T6SS). The three SS can translocate effectors to the human cell, where the most relevant targets are depicted. T4SS and T6SS can also target another bacterium. Taken from (Bleves et al., 2020)

1.2.1 Type IV Secretion Systems

T4SS are ancestrally related to transfer of DNA between bacteria, as part of the conjugative machineries. However, they are also linked to protein transfer to eukaryotic cells and pathogenicity, and to interbacterial killing. The T4SS has been classified in functional groups, according to their biological function (Figure 1.2): effector molecule translocation into host target cells; conjugation of chromosomal and plasmid DNA; DNA uptake and transformation; DNA release into the extracellular milieu (Backert & Meyer, 2006). More recently, T4SS has also been related with biofilm formation and killing bacterial neighbors (Souza et al., 2015) (Grohmann et al., 2018).

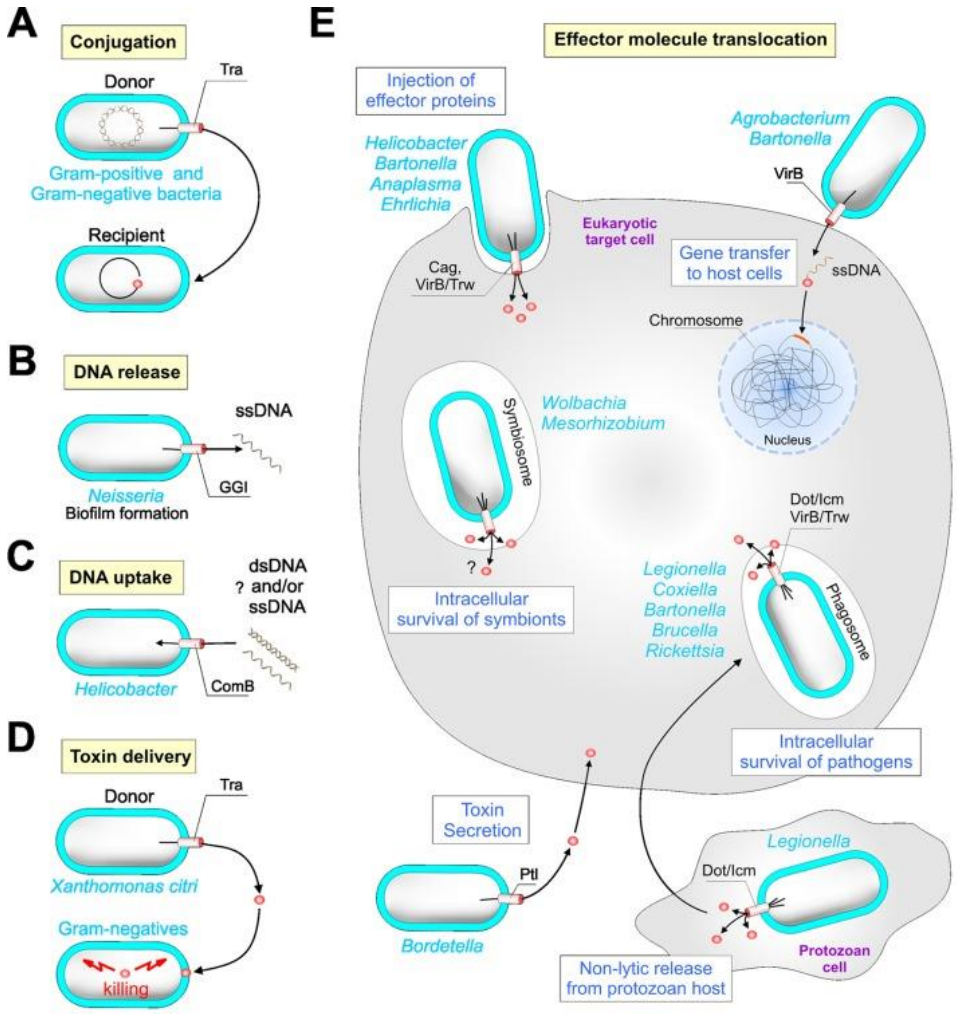

Figure 1.2 Schematic representation of T4SS functions in bacteria. Taken from (Grohmann et al., 2018)**.**

Despite the variety of functions, these systems evolutionarily share several elements in common in different species. There is a subfamily of T4SS with high similarity to the VirB/D4 from *A. tumefaciens* which is named T4ASS, while those with less similarity are classified as T4BSS (Alvarez-Martinez & Christie, 2009). The *Legionella pneumophila* Dot/Icm (Defective for organelle trafficking/Intracellular multiplication) system is the model used to study the T4BSS subfamily (Nagai & Kubori, 2011). The 3D structure of several of these T4SS has been elucidated (Fig. 1.3).

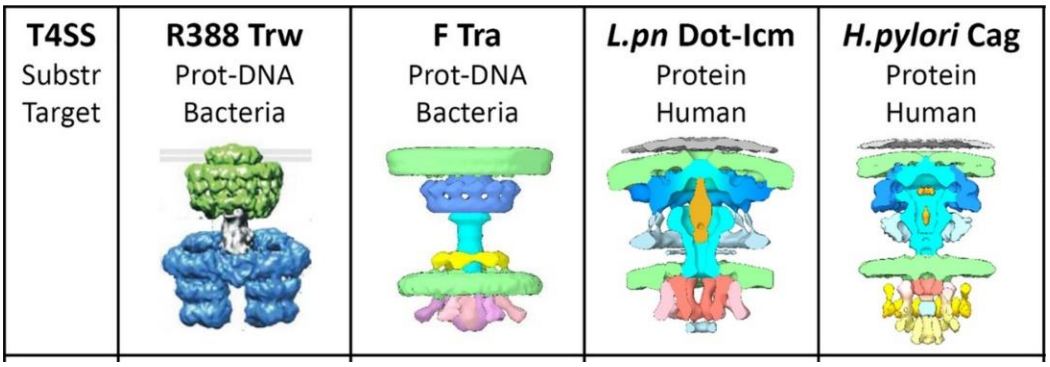

Figure 1.3 Comparison of the 3D structure of T4SS. The nature of the substrate and the target cell are indicated. Modified from (Bleves et al., 2020)

Most of T4ASS are made up of proteins named from VirB1 to VirB11, and VirD4. The T4SS encoded by *Escherichia coli* F plasmid, and the Cag (Cytotoxin associated genes) T4SS encoded by *Helicobacter pylori* are also classified as T4ASS due to the presence of VirB/VirD4 protein orthologues, although the sequence homology is limited. These systems require additional components in order to work properly (Backert et al., 2015). It is known that VirB2-VirB11 and VirD4 are required for substrate transfer, while VirB1 is necessary for assembly of conjugative pilus. Moreover, it was reported that VirD4 is dispensable for this assembly. According to their functions, these subunits can be grouped in four categories: the cytoplasmic ATPases (VirB4, VirB11, VirD4), components of an inner membrane platform (VirB3, VirB6, VirB8), constituents of an outer membrane core complex (VirB7, VirB9, VirB10) and pilus-assembly components (VirB1 transglycosylase, VirB2 pilin and VirB5 pilus-tip protein) (Grohmann et al., 2018). Figure 1.4 shows a schematic representation of how these proteins assemble into the T4SS.

Beyond the common functions among different T4SS, many components have acquired specialized functions for their particular substrate, function or target cell; For instance, VirD4 substrate receptors have acquired sequence variable C-terminal extensions that are capable to bind secretion chaperons specific for a determinate substrate. VirB6, has acquired large hydrophilic domains for interaction with the bacterial target cell. And VirB10 is implicated in specifying host cell recognition or immune evasion through its long variable repeat sequences (Christie, 2016).

The first 3D structure of a complete T4SS was reported for R388, a conjugative plasmid isolated from *E. coli* (Low et al., 2014). The structure is formed by a core complex of VirB6-VirB10 subunits, plus an inner membrane complex (IMC) that connects the core complex with

the cytoplasm. The IMC is composed of 12 copies each of VirB3, VirB4, VirB6 and VirB8, coming together to form a double barreled structure, each of the barrels protruding in the cytoplasm. These barrel shaped structures are each made of the VirB4 ATPase, observed as trimers of VirB4 dimers. VirB7, VirB9 and VirB10 proteins form a ring structure of 185 A°. Fourteen VirB10 subunits project each a helical bundle to form an outer-membrane channel. VirB4 and VirB11 are also essential for extracellular pilus formation (Grohmann et al., 2018).

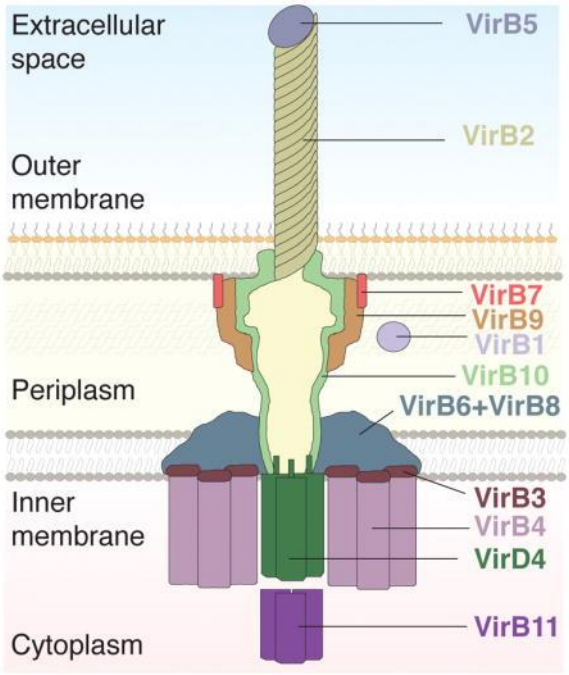

Figure 1.4 Schematics of a T4SS. Taken from (Grohmann et al., 2018)

Many T4SS are involved in conjugative DNA transfer. In this process, in addition to the T4SS, an essential protein is the relaxase, that initiates a nucleophilic attack of the active site tyrosyl hydroxyl group of the enzyme on the scissile phosphate group within *oriT*, releasing the bridging oxygen and forming a long-lived ssDNA-protein conjugate. This nucleoprotein complex is recruited by the T4SS and translocated to the recipient bacteria (Fig. 1.5). (Grohmann et al., 2018).

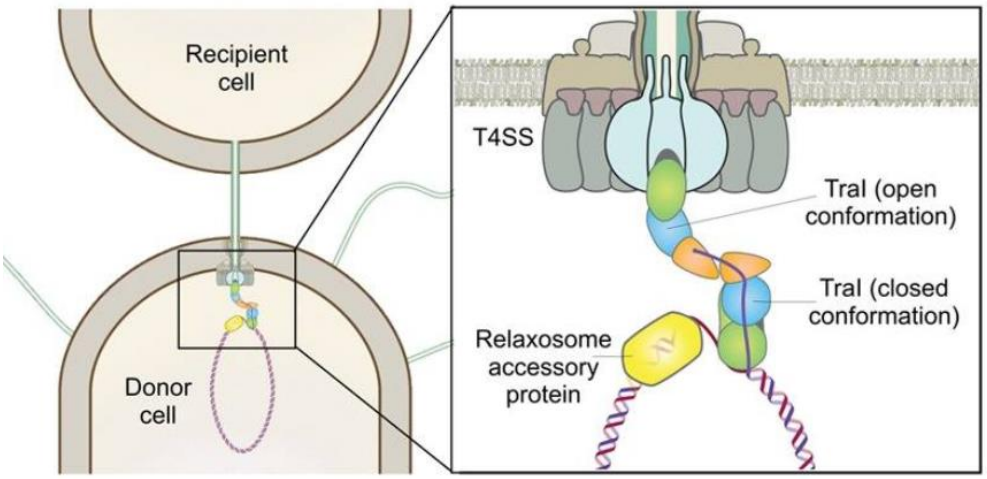

Figure 1.5 Bacterial conjugation process represented, where two relaxase monomers collaborate, adopting distinct structural conformations to provide the two necessary enzymatic activities for processing the DNA. Taken from (Grohmann et al., 2018)**.**

T4SS are versatile machineries. Recently, it was reported that T4SS of *Xanthomonas* species are specialized to transfer toxic bacterial effectors into rival bacterial cells, allowing bacterial competition. It was reported that this T4SS, chromosomally encoded, does not participate in DNA transfer, but it was related to bacterial killing (Souza et al., 2015) when *X. citri* was faced with *E. coli*. Bioinformatic analysis shows sequence homology of these genes with T4SS of other bacterial genera, such as Neiseriales and Burkholderiales (Sgro et al., 2019a).

1.2.2 Type VI Secretion Systems

T6SS are distributed widely in Barceriodetes and Proteobacteria, which include one third of the Gram-negative genera. They have been related to both interactions with eukaryotic cells acting as a virulence factor, as well as for competition between bacteria (Galán & Waksman, 2018). In fact, toxin production is one of the best-known examples of bacterial competition. T6SS inject toxins into the prey cell, with the aim of lysing it (Russell et al., 2014b). Interestingly, some toxins injected by T6SS have been shown to work as toxins both in prokaryotic and eukaryotic target cells (Cherrak et al., 2019b).

T6SS use a contractile mechanism to inject effectors into a target cell, either prokaryotic or eukaryotic. Fig. 1.6 depicts the structure of a T6SS. The injector consists of an inner tube covered by a herringbone complex that pierces the target cell membrane. The complex is built on an assembly platform called the base plate. The biogenesis begins with the assembly

of the membrane complex in the cell envelope and the base plate in the cytoplasm. Once the base plate is attached to the membrane complex, the inner tube and sheath are assembled in coordination (Cherrak et al., 2019b). T6SS is made up of thirteen components assembled as a large complex similar to an inverted bacteriophage tail. These components are anchored by ten membrane anchor copies (TssJ, TssL, and TssM) that function as the foundation of the system (Cherrak et al., 2019a). The assembled portion is connected to a transmembrane complex as an intracytoplasmic tube. This tube is composed of six Hcp hexamers surrounded by a contractile envelope assembled by two proteins, TssB and TssC. In its terminal part it is covered by two components, VgrG and PARA. This set of structures also works as effectors during the interaction of the system (Galán & Waksman, 2018). The toxin injection is driven by the shrinkage of the envelope.

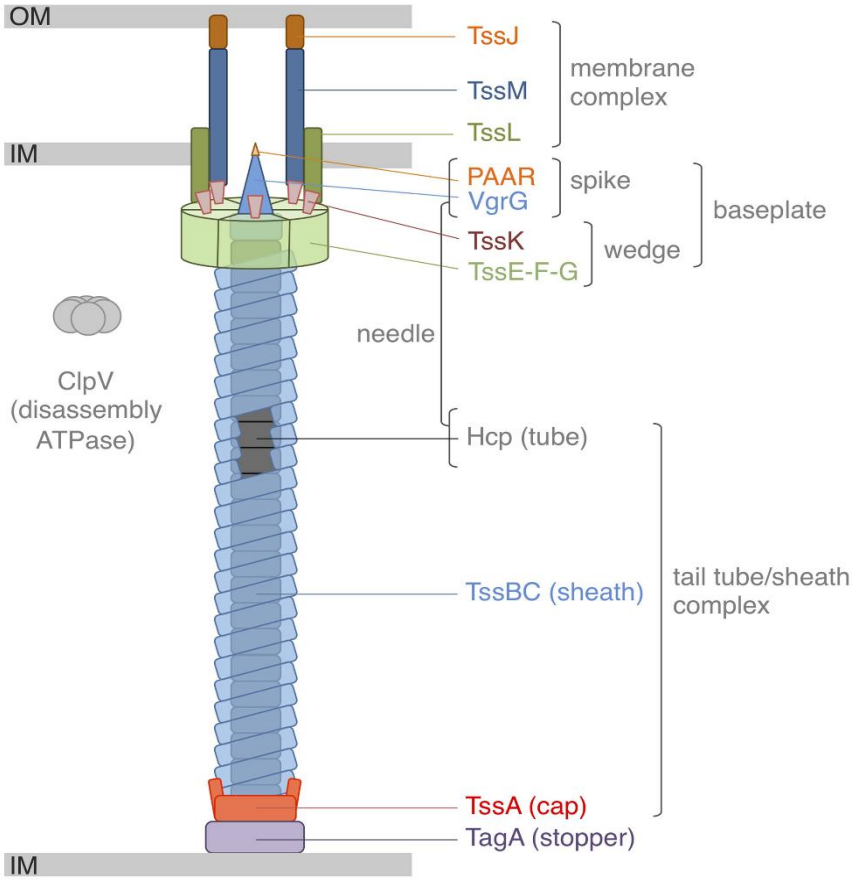

Figure 1.6. T6SS Structure taken from (Cherrak et al., 2019a)

1.3. *Burkholderia cenocepacia*

B. cenocepacia belongs to the *Burkholderia cepacia* complex, that contains 24 Gram-negative related bacterial species distributed in the environment, being pathogens both in plants and animals (De Smet et al., 2015). *B. cenocepacia* is a non-fermentative, Gram-negative species that lives as an opportunistic pathogen in patients with cystic fibrosis (CF), causing respiratory infections (LiPuma, 1998). CF is a common autosomal recessive illness in Europe affecting 1 in 2500 newborn (Farrell, 2008). The illness is produced due to mutations in the CFTR gene (cystic fibrosis transmembrane conductance regulator) that causes the production of viscous secretions in lungs (Knowles & Durie, 2002). These viscous secretions allow pathogens development and the subsequent infection that may lead to death (Ciofu et al., 2013).

B. cenocepacia infection treatment continues being challenging, due to the multidrug resistance of this bacterium (Alexander et al., 2008). This antibiotic resistance may be native or acquired. The first one is inherent to the microorganism, and is transmitted vertically. Whereas the second one is dependent on antibiotic selective pressure and HGT. There are many multidrug resistance mechanisms such as mutations in drug targets, transfer of resistance genes through phage mediated transduction and mobile plasmids, that may complicate *B. cenocepacia* treatment of the infections (Scoffone et al., 2017).

Precisely, HGT and microbial community formation in CF patients are fundamental aspects in order to understand the complexity of the illness. In fact, next generation sequencing based studies showed that pathogens such as *B. cenocepacia*, coexist with additional members of the CF lung community such as, *Stenotrophomonas maltophilia*, and *Achromobacter spp*., *Mycobacterium abscessus* complex, *Mycobacterium avium* complex, and the *Streptococcus milleri* group, that may contribute to the disease due to the establishment of complex interspecies interactions that limit the efficacy of antibiotic treatments (Scoffone et al., 2020) (Vandeplassche et al., 2019).

Multiple *B. cenocepacia* virulence factors may be other reason that complicate treatment of its infection. Virulence factors include bacterial adhesins, colonization factors, protein toxins like hemolysins, and molecules that affect the innate and adaptive immune responses (Zachary, 2017). In *B. cenocepacia* many virulence factors have been reported related to secretion systems usage. Table 1.1 shows an overview of these virulence factors.

Virulence factor	Features	Refs
Exopolysaccharide	Phagocytosis and reactive oxygen species (ROS) production interference	(Leitão et al., 2017)
Biofilm formation	Enhanced in the presence of neutrophil-like dHL60 cells; bacterial protection from recognition by the immune system	(Murphy & Caraher, 2015)
Lipopolysaccharide	Infection establishment; neutrophil respiratory burst response and stimulation of the production of proinflammatory cytokines	(Khodai-Kalaki et al., 2015; Leitão et al., 2017)
Secretion systems	T2SS: zinc metalloprotease secretion T4SS-1 and T3SS: role in intracellular survival T5SS: useful for bacterial adhesion T6SS: affects the actin cytoskeleton of macrophages; activates the inflammasome and enhances the activity of caspase-1	(Leitão et al., 2017)
Siderophores	Ornibactin, pyochelin, cepabactin, and cepaciachelin: iron chelation and uptake during infection	(Butt & Thomas, 2017)
Flagellin	Infection establishment	(Kumar & Cardona, 2016)

Table 1.1 *B. cenocepacia* Virulence Factors taken from (Scoffone et al., 2020)

In *B. cenocepacia,* secretion systems have been studied due to their importance both in pathogenicity and microbial interactions (Leitão et al., 2017). For example, T2SS is involved in secretion of two zinc metalloproteases, ZmpA and ZmpB, which play a role in virulence in a rat agar bead chronic infection model (Kooi et al., 2006). In a similar type of assay, T3SS seems to play a role in host immune system evasion, being important for pathogenesis in lung murine model (Tomich et al., 2003). However, it also seems to play no role in intracellular survival in murine macrophages model (Lamothe et al., 2007). Two T4SS have been described in *B. cenocepacia.* The Ptw (Plant Tissue water soaking) pT4SS encoded in a native plasmid of 92 kb, and the VirB/D4 T4SS located in chromosome II (Engledow et al., 2004). Their role in virulence is controversial. Finally, the T6SS has been involved in both host interaction and bacterial competition.

1.3.1 The VirB/D4 T4SS

The VirB/D4 T4SS is located on chromosome II and bears homology to the VirB/D4 T4SS of *A. tumefaciens* (Engledow et al., 2004). Sequence analysis of this region revealed a lower G+C content (63%) compared with the G+C content of the entire genome (66.9%), suggesting acquisition through HGT. Two genes, designated BCAM0322 and BCAM0323, upstream of the VirB/D4 system showed homology to a two-component regulatory system (Fig. 1.7). It has been reported that this T4SS is not involved in the Ptw phenotype, rather, it seems to participate in plasmid mobilization (Zhang et al., 2009). A gene designated *bcvirD4* showed homology to other T4SS coupling proteins, which serve as active motors necessary

for substrate transfer through the T4SS (O'Grady, 2011). It has been determined that the RSF1010 transfer intermediate and Osa Fertility inhibitor suppress *A. tumefaciens* oncogenesis specifically by interfering with T-DNA and VirE2 substrate binding to the VirD4 receptor (Cascales et al., 2005). In *B. cenocepacia*, Osa interfered with the mobilization of pML122Tc, indicating its similar function with VirD4 in *A. tumefaciens* and its implication in conjugation (Zhang et al., 2009). Expression of the T4SS *vir* genes on the chromosome 2 were similar in the *in vitro* and *in vivo* conditions (rat agar bead respiratory infection models) suggesting this T4SS has no role in infection of mammalian cells (O'Grady, 2011).

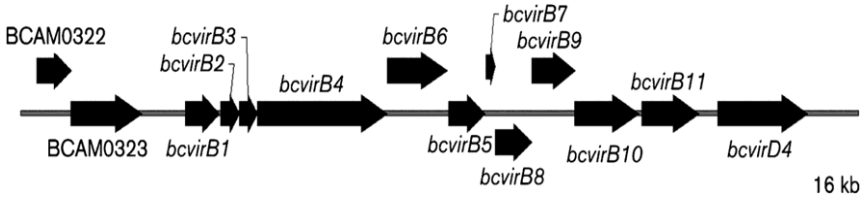

Figure 1.7. Schematic representation of the VirB/D4 T4SS locus on chromosome II of *B. cenocepacia* strain J2315. Designation of genes was based on homology to gene products of transfer- and translocation-related proteins. Genes are represented as solid boxes with arrowheads indicating their orientation Taken from (Zhang et al., 2009).

1.3.2 The Ptw pT4SS

Ptw pT4SS was named due its involvement on the secretion of a plant cytotoxic protein(s) that causes plant tissue watersoaking phenotype. This phenotype may be described by the presence of a droplet on the onion surface when it is inoculated with *B. cenocepacia* (Engledow et al., 2004). It was reported that insertions or deletions in *ptwD4*, abolished the watersoaking phenotype, presumably by affecting protein effector translocation (Engledow et al., 2004). Thus, this T4SS was proposed to be related with plant virulence of *B. cenocepacia*. In fact, other *Burkholderia* species which do not interact with plants, such as *B. vietnamiensis* G4 and *B. xenovorans* LB, did not reveal the presence of *ptw* homologs. According to Zhang et al., Ptw pT4SS is not related with conjugative DNA transfer. They claimed the system does not contain components necessary to support conjugation, and in particular no *oriT* homolog, suggesting that functionally it is a member of the effector–translocator subfamily of T4SSs (Zhang et al., 2009).

It has been reported that plasmid-encoded Ptw pT4SS plays a role in the intracellular survival of *B. cenocepacia* in both professional and non-professional phagocytes. A functional pT4SS contributes to the ability of *B. cenocepacia* to evade endocytic degradation,

and to survive and replicate in both airway epithelial cells and monocyte-derived macrophages (Sajjan et al., 2008). The level of expression of the genes encoded in this plasmid seems to be related with the media where they are being expressed. In a rat infection model, it has been demonstrated that expression was markedly induced *in vivo* at levels ranging from 3- to 46.1-fold higher than the in vitro growing conditions. This might suggest an implication of these genes in the interaction with the eukaryotic host (O'Grady, 2011). These results were confirmed by our laboratory, where we observed a drastic increase in the expression driven by the Ptw promoter region in the presence of onion extract (Fernández-González et al., 2016).

In contrast, M. Valvano reported that there are no differences in intracellular survival in murine macrophages measured as recovered bacteria at 24 h post infection in *B. cenocepacia* strains with Ptw mutations. Therefore, it is possible that one or more secretion systems in *B. cenocepacia* will be needed for functions such as survival in different tissues or establishment of infection in non-mammalian hosts (Valvano, 2015).

On the other hand, our own detailed bioinformatics analysis showed that the Ptw pT4SS is a chimera composed of VirB/D4 and F-specific subunits, encoding a putative relaxase and other proteins required for conjugative DNA processing (Fernández-González et al, 2016). Our results proved that the Ptw plasmid encodes a conjugative system, that mediates horizontal transfer of DNA between bacteria. It was demonstrated that upstream the coupling protein gene, there was an *oriT* region. Even though conjugation of the Ptw plasmid itself could not be detected, it was proved that a part of the Ptw plasmid, identified as a DNA transfer region, was conjugatively transferred from *B. cenocepacia* to *B. cepacia* strains (Fernández-González et al., 2016).

In summary, the role of Ptw pT4SS remains unclear, since it can mediate DNA transfer among bacteria, but the *ptw* genes are expressed upon contact of the eukaryotic host, and there are also evidences, although controversial, for a contribution to intracellular survival.

1.3.3 The T6SS

T6SS have been involved in bacterial competition in various microorganisms such as *Pseudomonas aeruginosa*, *Vibrio cholerae*, and *Serratia marcescens* (Spiewak et al., 2019). In the *Burkholderia* genus, eight different T6SSs have been identified, but *B. cenocepacia* strains contain only a single T6SS that corresponds to T6SS-1 of *B. pseudomallei* and *B. thailandensis* (Angus et al., 2014; Aubert et al., 2015). This T6SS has been reported to contribute to *B. cenocepacia* survival in a rat model of chronic lung infection. Its effect may be due to the deregulation of the Rho family of GTPases, linking

disruption of the actin cytoskeleton and delayed NADPH oxidase activation (Aubert et al., 2015; Flannagan et al., 2012; Rosales-Reyes et al., 2012)(Aubert et al., 2016). The T6SS also seems to be downregulated by *atsR*, interfering with caspase-1 activation, causing morphological changes in murine macrophages by actin cytoskeleton modification (Aubert et al., 2015). Interestingly, T6SS also mediated T2SS effectors secretion into the host cytoplasm, such as the metalloproteases ZmpA and ZmpB, conferring a role for T2SS in intracellular survival and replication of *B. cenocepacia* (Leitão et al., 2017).

On the other hand, a recent report proves that *B. cenocepacia* T6SS is also involved in bacterial competition, as shown by a series of bacterial competition assays against *Pseudomonas putida* and *E. coli*. In this study it is also suggested that *B. cenocepacia* T6SS is not required for virulence in *Caenorhabditis elegans, Galleria mellonella,* and zebrafish embryo eukaryotic models (Spiewak et al., 2019).

2. Hypothesis

Previous results of several laboratories suggested that the Ptw pT4SS played a role in the infection of the eukaryotic host, delivering effectors responsible for the watersoaking phenotype and contributing to *B. cenocepacia* survival inside macrophages. Results from our laboratory, support a dual function for Ptw pT4SS, as a pathogenic T4SS, inducing its expression when in contact with the onion tissue, and as a conjugative T4SS, mediating horizontal transfer of the Ptw plasmid between bacteria. Altogether, the possibility existed that the Ptw pT4SS could be involved in DNA transfer to the eukaryotic host, similar to the *Agrobacterium* T4SS-mediated DNA transfer process to plant cells.

Little is known about the role of other secretion systems in *B. cenocepacia*, including the VirB/D4 T4SS. We decided to test a yet unexplored possibility: the involvement of either T4SS in bacterial killing. Moreover, it´s known that T4SS activity may affect T6SS-mediated antibacterial activity, by encoding regulatory elements for controlling its activity. In the present study, we set up a bacterial killing assay between *B. cenocepacia* mutants and *E. coli* in order to explore the role of T4SS and T6SS in this process.

3. Objectives

Our objectives are to test a collection of *B. cenocepacia* mutants lacking different secretion systems for the following phenotypes:

1. Plant tissue watersoaking on onion slices.
2. Establish a Bacterial Killing Assay to test *B. cenocepacia* killing of *E. coli*.
3. Assess the conjugative DNA transfer ability of Ptw pT4SS.
4. Intracellular survival of *B. cenocepacia* in cultured macrophages
5. DNA transfer from *B. cenocepacia* to cultured human cells

With this approach, we expect to get insight on the role of the different *B. cenocepacia* secretion systems in horizontal transfer in bacterial communities, bacterial competition and virulence.

4. Material and methods

4.1.Bacterial strains

The *B. cenocepacia*, *Burkholderia cepacia* and *E. coli* strains used in this work are listed in Tables 4.1 and 4.2.

Table 4.1. *B. cenocepacia* and *B. cepacia* strains

Strain[1]	Genotype[2]	Reference/Source
B. cenocepacia		
K56-2	ET12, Toronto, Canada, CF	(Darling et al., 1998)
J2135	ET12, Edinburgh, UK, CF	(Govan et al., 1993)
J2135 ΔPtw	J2135 cured of plasmid Ptw	From A. Vergunst Lab
JST17 ΔpT4	MHK1 ΔpBCA017-059	From Valvano Lab.
JST39 ΔT4	MHK1 ΔBCAM0324-35	From Valvano Lab.
JST52 ΔpT4 ΔT6	MHK1 ΔBCAL0333-52, ΔpBCA017-059	From Valvano Lab.
JST150 ΔT2 ΔT3 ΔT6	MHK1 ΔgspDEF, ΔBCAM2040-57, ΔBCAL0333-52	From Valvano Lab.
MHK1	K56-2 ΔBCAL1674-6 Gm-sensitive	(Hamad et al., 2010)
B. cepacia		
CECT 322	*B. cepacia* isolated from exforest soil, Seven Mile Strech, Trinidad	ATCC

[1] ΔTX refers to the type of secretion system deleted. To distinguish between the two T4SS, pT4 will refer from now on to the Ptw T4SS codified in the plasmid.
[2] CF, isolated from cystic fibrosis patient.

Table 4.2. *E. coli* strains.

Strain	Genotype	Reference
D1210	Sm^R, *recA hspR hsdM rpsI lacI*^{q}	(Sadler et al., 1980)
DH5α	Nx^R, *F' endA1 hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA) U 169 80dlac Δ M15*	(Grant et al., 1990)

4.2.Plasmids used in this work

Table 4.3 Plasmids used in this work.

Plasmid	Phenotype	Description	Reference
pBBR6	Gm^R	Cloning vector derived from pBBR1-MCS	(Vergunst, 2000)
pSU1445	Km^R Tp^R	R388::Tn5tac in *trwC*	(Llosa et al., 1994)
pEF22	Cm^R	pBBR1::*oriTptw* + *ptwA* + 1/3*ptwD4*	(Fernández-González et al., 2016)
pEF31	Cm^R	pBBR1::*oriTptw* + *ptwA* + *ptwB* + *ptwC*	(Fernández-González et al., 2016)

4.3.Molecular biology techniques

4.3.1. DNA extraction and Purification

GenElute Plasmid Miniprep Kit (Sigma) was used for general plasmid isolations. Gen Elute PCR Clean-Up Kit (Sigma) was used for purification of PCR products. GenElute Agarose Spin Columns (Sigma) were used for DNA purification from Agarose gels. Total DNA from *E. coli* and *B. cenocepacia* was extracted using Instagene Matrix (BioRad) following manufacturer´s instructions. The concentration of DNA samples was determined with a Nano-Drop Spectrophotometer ND-1000.

4.3.2. PCR amplification

DNA amplifications were performed using Kapa Taq DNA polymerase (Biosystems). A iCycler (BioRad) thermocycler was used with the following program: 1 min denaturation at 95°C, 30 cycles of amplification (30 seconds at 95°C of denaturalization, 60 seconds of the annealing temperature at 49 °C, and 30 seconds per kb to be amplified of elongation time at 72°C) and a last step of 5 min of extension at 72°C. After this, samples were maintained at 4°C.

4.3.3. Agarose gel electrophoresis

PCR amplification products were analyzed by agarose gel electrophoresis. Agarose was dissolved in TBE 0.5X (Tri-HCl 45 nM, boric acid 45 mM, EDTA 0.5 mM and pH 8.2) to a concentration of 1-2% w/v, as needed according to the size of the DNA fragments to be resolved. SYBR Safe (Invitrogene) was used at a concentration of 1:100.000. Loading buffer (0.25% bromophenol blue (w/v), 30% glycerol (v/v) in TBE 0.5X) was added to the samples in a 5:1 relation of DNA sample volume/ blue dyer. GeneRuler 1 kb DNA Ladder (ThermoScientific) was used as a molecular weight marker. A horizontal BioRad electrophoretic system was used (80-120 V). Agarose DNA gels were visualized with a ChemiDoc™ XRS+ System (BioRad), and images were analyzed with Quantity One software (BioRad).

4.3.4. RT-PCR (Planned)

RNA from 2 ml of bacterial culture (in different stages of the growth curve) will be extracted using RNeasy Protect Bacteria Kit (Qiagen) following manufacturer's instructions and subsequently treated with DNAse (New England Biolabs). RT-PCR will be done in two steps; first a Reverse Transcription of 1 pg of RNA to obtain the cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche) following manufacturer's instructions and a second step, in which PCR will be done using 25 ng of cDNA and the SYBR Green I Master (Roche) on a LightCycler 480 LightCycler® 480.

4.4.Microbiological techniques

4.4.1 Growth conditions and selection media

Handling of P2 pathogenic strains was carried out in a P2 biosafety laboratory, following the P2 safety regulation procedures.

E. coli

Luria-Bertani (LB) broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) was used for bacterial growth, supplemented with agar 1,5% (w/v) for solid culture. Cultures were incubated at 37°C. Selective media included antibiotics (Sigma, Apollo) at the following concentrations: ampicillin (Ap), 100 μg/ml; chloramphenicol (Cm), 50 μg/ml; kanamycin monosulphate (Km), 25 μg/ml; nalidixic acid (Nx), 20 μg/ml; gentamycine sulphate (Gm), 10 μg/ml; trimethoprim (Tp), 20 μg/ml. X-gal (5-bromo-4-cNoro-3-indolyl-beta-D-galactopyranoside) was supplied at a concentration of 40 μg/ml. To preserve *E. coil* strains, a stationary phase culture was centrifuged and resuspended in 50% peptone 1,5% (w/v) and 50% glycerol. Duplicates of all the strains were kept at -20°C and -80°C.

Burkholderia

B. cenocepacia and *B. cepacia* were grown under the same conditions as *E. coli*. Antibiotics were used as follows: Cm 100 μg/ml; Tp 250 μg/ml. To preserve *B. cenocepacia* and *B. cepacia,* 800 μl of an o/n culture are mixed with 200 μl of 75% glycerol and stored at -80°C, since they cannot be preserved at -20°C.

4.4.2 Bacterial conjugation

Donor (*B. cenocepacia* harboring the indicated plasmids) and recipient (*E. coli (*pSU1445)) strains were grown overnight at 37°C with appropriate antibiotic selection (Cm100 for *B. cenocepacia* and Tp20 for *E. coli* (pSU1445)). Matings were performed in solid media by mixing 500 μl both the donor and the recipient strains. Bacterial cultures were adjusted to an OD_{600}=0.5, mixed at an equal radio, washed twice, and transferred to a conjugation filter (0,22 μm nitrocellulose, Sartorius) on a LB agar plate. After 18h incubation, dilutions were plated on selective media for donors, recipients and transconjugants. Transconjugants were plated in (Cm100 + Tp250). The frequency of conjugation was expressed as the number of transconjugants per donor cell. When indicated, onion extract (10 μl/ml, prepared as described in Section 4.6.2) was adder to donor strain culture to induce *ptw* expression during the overnight grow. The conjugation filter was placed on an LB agar plate supplemented with onion extract.

4.4.3 Bacterial Killing Assay

We used the protocol of (Spiewak et al., 2019). Killer (*B. cenocepacia*) and prey (*E. coli*) strains were grown separately overnight in LB at 37°C. Each culture was then normalized to an OD_{600} of 0.5. Bacterial suspensions were combined in a 5:1 killer: prey

ratio. Monoculture controls of prey and killer strains with LB were included using the same number of bacteria as in the killer: prey mixture, respectively. In order to accomplish this, three preparations were made. The first one corresponds to killer strain and consisted on 500 µl of killer strain and 100 µl of LB medium. The second mixture corresponds to the prey strain and consisted on 500 µl of LB medium and 100 µl of prey strain. The third preparation corresponds to the killer: prey mixture and consisted on 500 µl of killer and 100 µl of prey strain.

Then, 25 µl of each preparation were spread over a 0.45-µm nitrocellulose filter membrane (Sartorious) on a prewarmed LB agar plate and incubated at 37°C for 4 h. After incubation, bacteria from each filter membrane were harvested in 2 ml LB and 10^{-1}, 10^{-3}. 10^{-4} and 10^{-5} dilutions were made. 100 µl of each dilution were spotted onto selection plates in duplicate. It was repeated for each mutated strain in this assay.

The killer *B. cenocepacia* K56-2 was selected by Ap100 resistance, and the prey *E. coli* (pBBR6) by Gm10 resistance in K56-2 preparation and byNx20 resistance in J2315 preparation Plates were incubated at 37°C overnight. The number of viable CFU was counted and used to calculate the prey survival percentage for each mixture, referred to the control prey-only culture, which was considered 100%. All experiments were carried out at least three times.

4.5 Cellular biology techniques (Planned)

4.5.1 Cell culture

Human cell lines are listed in **Table 4.4.**

Table 4.4 Human cell lines.

Cell line	Description	ATCC NAME
IB3-1	Human CF Bronchial epithelial cells	CRL-2777
HeLa	Epithelial Human cells of cervix adenocarcinoma	CCL-2

Cells are grown in DMEM with Glutamax (Gibco) supplemented with 10% fetal bovine serum (FBS) (Cambrex) at 37 °C under a 5% CO_2 atmosphere. Flasks for IB3 culture are pre-coated with a coating solution (RPMI + 10% FBS, Collagen Solution Type I (Sigma), Fibronectin from bovine plasma (Sigma) and BSA (Sigma)), essential for the IB3 growth dispersion.

4.5.2 Infections of mammalian cells

IB3-1 infections start by seeding approximately 50,000 cells per well. In order to calculate the MOI, previous experiments recommend us to consider that an OD_{600} of 1 equals 1.2 x 10^8 bacteria per ml. A MOI of 10 is recommended. *B. cenocepacia* is diluted in DMEM. Then, cells are washed with PBS. Bacteria are added to the cells. The infections occur during 2-20 h at 37 °C under 5% CO_2. Afterwards, cells are washed twice with PBS, and incubated with DMEM supplemented with Gm10 to kill extracellular bacteria.

4.5.3 Determination of the number of intracellular bacteria

In order to quantify intracellular bacterial survival and growth, infection mixtures are incubated for different periods of time (2, 24, 48, 72h) and washed with Gm10 to kill the extracellular bacteria. After 2 PBS washes, human cells are lysed by osmotic shock with water for 15 min at 40°C or adding trypsin-EDTA 4X. Then, serial dilutions are plated in LB to count the number of intracellular bacteria. The last PBS wash is also plated to check that no extracellular bacteria are alive after the antibiotic treatment.

4.5.4 Fluorescence microscopy

This technique is used to check the morphology of infected cells, GFP expression upon DNA transfer from bacteria, and to follow the infection process using red fluorescence bacteria. A Nikon Eclipse E400 microscopy is used. Filters used for excitation and emission spectra are 488 nm and 530 nm to look for eGFP positive cells and 510 nm and 590 nm for DsRed.

4.5.5 Flow cytometry

Flow cytometry is used to detect and quantify human cells expressing eGFP after the infection with the bacterial pathogens. After the infection, the supernatant is removed. The cells are washed with PBS two times and then trypsinized. After centrifugation, the pellets are resuspended in 200-300 µl of PBS in special tubes for flow cytometry (Deltalab). Each sample is analyzed in a Cytomics FC50 flow cytometer (Beckman Coulter).

To quantitate the results, first of all, the viable cell population is established, eliminating attached cells, dead cells and bacteria, by means of size and complexity. Subsequently, the eGFP or DsRed background line is defined with uninfected cells, delimiting the start point of the P2 population of eGFP or DsRed positive cells.

4.6 Plant assays

4.6.1 Plant watersoaking assay

The Ptw assay was performed as described by Engledow et al. (Engledow et al., 2004). All experiments were performed with white onions previously washed in sterile water during 30 minutes. Bacterial suspensions of *B. cenocepacia* strains were adjusted to OD_{600}= 0.5 and individual onion scales were wounded on the axial (inner) surface with a sterile blade. 10 μl of bacterial suspension was inoculated into the wound. *E. coli* DH5α was inoculated as a negative control. Onion scales were placed on a petri dish containing Whatman paper towels premoistened with sterile distilled water, sealed, and incubated at 37 °C. Ptw activity was estimated by the appearance of water drops on the onion tissue at 24 hours post inoculation.

4.6.2 Preparation of onion extract

Onion extract was obtained squeezing onions and the extract was centrifuged at 10.000 rpm for 5 min three times. Supernatant is then sterilized by filtration through 0,22 μm filter (Sartorius) and added directly to the bacterial growth media.

4.7 Bioinformatics programs and databases

NCBI Database: The NCBI is comprised of a series of databases relevant to biotechnology and biomedicine. It includes major databases as GenBank for DNA sequences and PubMed, a bibliographic database for the biomedical literature. NCBI databases were used to look for complete genomes and annotations of *B. cenocepacia* (https://www.ncbi.nlm.nih.gov).

BLAST: Basic Local Alignment Search Tool finds regions of similarity between biological sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance. Megablast is intended for comparing a query to closely related sequences and works best if the target percent identity is 95% or

more. Discontiguous megablast uses an initial seed that ignores some bases (allowing mismatches) and is intended for cross-species comparisons. BlastN allows a word-size down to seven bases. (https://www.blast.ncbi.nlm.nih.gov).

In the present work, BlastN was used to compare the sequence corresponding to *B. cenocepacia* J2315 pT4SS, T4SS and T6SS against the entire group of *B. cepacia* complex (Bcc). Genes pBCA017 to pBCA059 in accession number AM747723.1 were used for pT4SS analysis. Genes BCAM0324 to BCAM0335 in accession number AM747721 were used for VirB/D4 T4SS analysis. Genes BCAL0333 to BCAM0352 in accession number AM747720.1 were used for T6SS analysis.

A graphic with the homology results was created, percentage of relative homology was calculate using a highly conserver genetic region F0F1ATP (BCAL0024 to BCAL0041) as a control. Number of blast hits obtained was divided to the number of Kb, The F0F1 ATP result of this operation, was considered as 100% of relative homology. RPS genetic region was also included as a positive control (BCAL0219 to bcal0264).

Graph Pad Prism 8: Graph Pad was used for the construction of the graphs using the data obtained from the experiments. It is a commercial scientific 2D graphing and statistics software available at: (https://www.graphpad.com)

4.8 Schedule

Activities	Year																															
	In charge	Month 1				Month 2				Month 3				Month 4				Month 5				Month 6				Month 7				Month 8			
		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
1. Bibliographic review	Student				*																											
2. Lab. training	Student																															
3. P2 Training	Student																															
4.Conjugation assays	Student																															
5. Plant Assays	Student																															
6. Competition Assays	Student																															
7. Celular culture assays	Student																															
8. DNA Transfer assays	Student																															
9. TFM writing	Student																															
10. TFM revision	Tutor																															
11. TFM presentation	Student																															

* Green colored squares correspond to worked already done. Blue squares correspond to future planned work.

5. Results

5.1 Bioinformatic Analysis of Secretion Systems in *Burkholderia cepacia complex*

Bioinformatic analysis were made using BLASTN algorithm in order to evaluate the conservation of T4SS, pT4SS and T6SS from *B. cenocepacia* J2315 in the Bcc group. The *B. cenocepacia* J2315 whole VirB/D4 T4SS sequence was compared with the gene database of Bcc. The first 17 hits with a high homology (>95%) comprise species from *B. cepacia* DDS 7H-2 to *B. cenocepacia* CR318. Then, the following 29 hits with fragmented homology (67% to 72%) were found. This group comprises species from *B. ubonensis* to *B. contaminans*. (Fig 5.1). These results indicate a high conservation of this T4SS in *Burkholderia cepacia complex*.

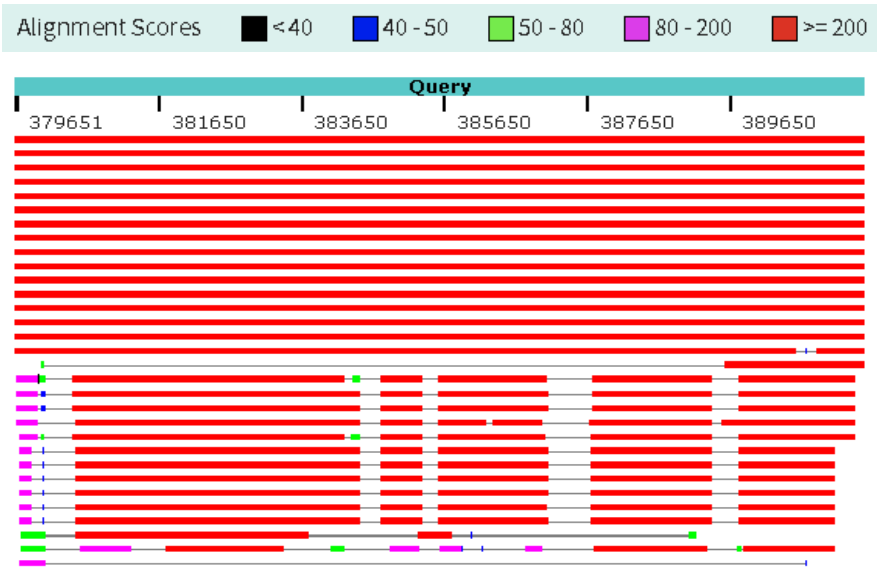

Fig 5.1 Distribution of top BAST hits between *B. cenocepacia* VirB/D4 T4SS and Bcc

Then, *B. cenocepacia* J2315 whole pT4SS sequence was compared with the gene database of Bcc (Fig. 5.2). Only 4 medium-high homology hits (>64%) were found, from a 95,35 % homology in *B. pyrrocinia*, to 73,67 % in *B. ubonensis*. These results indicate a low conservation of the pT4SS in Bcc.

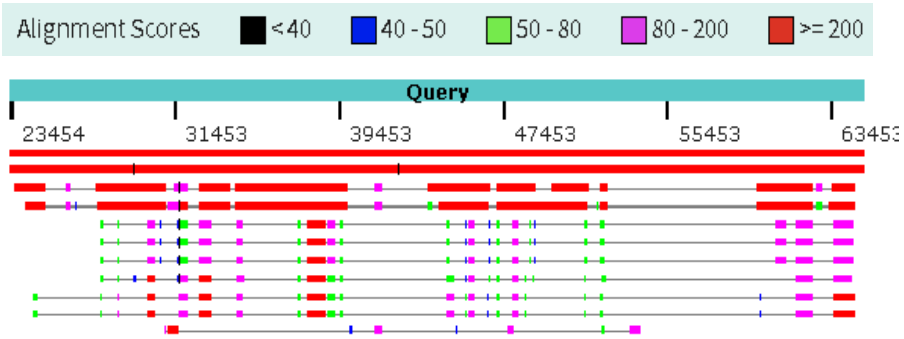

Fig 5.2 Distribution of top BAST hits between *B. cenocepacia* pT4SS and Bcc

Then, the *B. cenocepacia* J2315 whole T6SS sequence was compared with the gene database of Bcc. 93 high homology hits (>94%) were found (Fig. 5.3), from a 99,67 % homology in *B. cepacia*, to 94.31% in *B. pyrrocinia*, or 94.57% in *B. stabilis*. This result indicates a high conservation of the T6SS in Bcc.

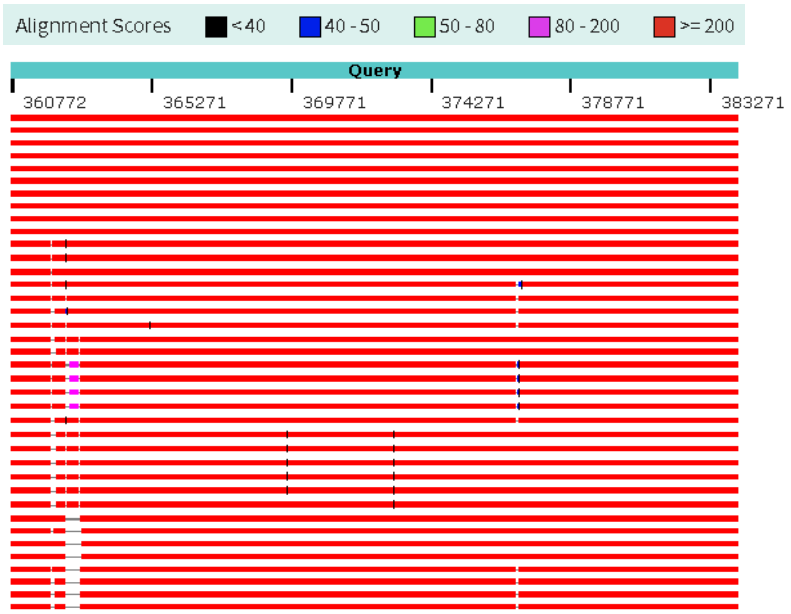

Fig 5.3 Distribution of top BAST hits between *B. cenocepacia* T6SS and Bcc

Finally, we have analyzed two genetic regions, F01F ATP and RPS, that have been reported to be highly conserved in Bcc (Juhas et al., 2012). These regions were used as a positive control for T4SS, pT4SS, and T6SS homology comparison (Table 5.1/ Fig 5.4).

Table 5.1 Conservation of T4 and T6SS in Bcc

***B. cenocepacia* Genetic Region**	**Size (kb)**	**Blast Hits > 70%****	**Blast hits / kb**	**% Homology / F01F ATP**
F01F ATP*	21	93	4,4	100
RPS*	40	93	2,4	54
T6SS	23	95	4,1	92
T4SS	12	31	2,6	59
pT4SS	42	11	0,3	6

*F01F ATP and RPS are 2 genetic regions highly conserved in Bcc.
**BLAST between *B. cenocepacia* and Bcc genebank

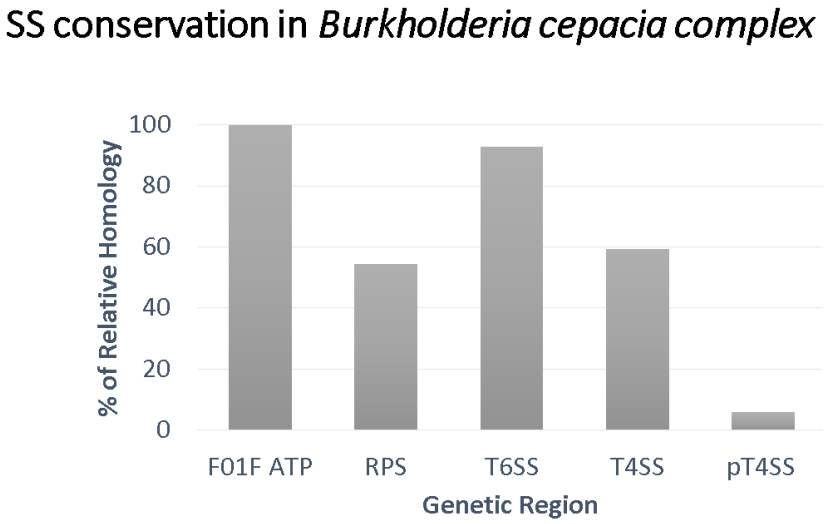

Fig 5.4 Secretion Systems Conservation in Bcc. Graphic represents the percentage of Relative Homology, to the highly conserved genetic region F01F ATP (100% of relative homology).

5.2 Conjugation Assays

Previous results carried out in our laboratory, showed that part of the putative conjugation machinery of the plasmid Ptw could be mobilized by conjugation (Fernández-González et al, 2016). These results proved the existence of an origin of transfer in this region, and strongly suggest that PtwC, a homolog of the conjugative relaxase TrwC, is involved in conjugative DNA transfer. However, it is important to remark that conjugation of the native Ptw plasmid itself was not shown. Therefore, the described experiments were performed detecting the conjugation of a co-resident plasmid pEF31 that contained the Ptw region homologous to the R388 Dtr region (Fernández-González et al., 2016). This plasmid was mobilized from *B. cenocepacia* to *B. cepacia* and the mobilized product was confirmed by PCR in the transconjugants.

Our aim was to confirm these results by repeating the assays, and subsequently, to use newly-acquired *ptw* mutant strains from other laboratories (namely, the strain J2315 cured of the Ptw plasmid), in order to confirm the involvement of the pT4SS in conjugative DNA transfer. Matings were carried out, under the conditions described in Materials and Methods, using as donor strains K56-2 (pEF31) or J2315 (pEF31) and as recipient strain *B. cepacia* CET 322 (pSU1445). As a negative control we used pEF22; this plasmid does not encode *ptwC* and thus it does not produce the relaxase PtwC in *cis*, a requirement for plasmid mobilization (Fernández-González et al, 2016). We were not able to reproduce the conjugation results previously reported (Table 5.2), not even adding onion extract to induce ptw expression (Tables 5.3). In the assays using J2315 as donor, some colonies were obtained, but after replica-analysis, it was concluded that they were donors which became resistant to Tp, not transconjugants. Spontaneous antibiotic resistance is frequent in *Burkholderia*. It should be highlighted that both, the donor and the recipient strain were recovered in good amounts, ruling out the lack of bacteria as a possible explanation for the absence of transconjugants.

Table 5.2 Conjugation assays from *B. cenocepacia* to *B cepacia* CET322 (pSU1445)

Donor strain	Donors * (Cm100)	Recipients* (Tp250)	Transconjugants *(Cm100 Tp250)	Conjugation Frequency**
K56-2 (pEF31)	45 (-6)	49 (-6)	<1 (0)	$< 2{,}2 \times 10^{-8}$
K56-2 (pEF22)	59 (-6)	40 (-6)	<1 (0)	$< 2{,}5 \times 10^{-8}$
J2315 (pEF31)	95 (-6)	900 (-4)	<1 (0)	$< 3{,}2 \times 10^{-8}$
J2315 (pEF22)	79 (-6)	49 (-6)	<1 (0)	$< 2{,}2 \times 10^{-8}$
J2315 ΔPtw (pEF31)	118 (-6)	37 (-6)	<1 (0)	$< 8{,}47 \times 10^{-9}$

*Number of colonies per plate. Parenthesis shows the dilution log.
** Transconjugants per donor

Table 5.3 Conjugation assays from *B. cenocepacia* to *B cepacia* CET322 (pSU1445) in the presence of onion extract

Donor strain	Donors* (Cm100)	Recipients* (Tp250)	Transconjugants (Cm100 Tp250)	Conjugation Frequency**
K56-2 (pEF31)	77 (-6)	117 (-6)	<1 (0)	$< 1{,}30 \times 10^{-8}$
K56-2 (pEF22)	1000 (-6)	800 (-6)	<1 (0)	$< 1{,}00 \times 10^{-9}$
J2315 (pEF31)	800 (-6)	240 (-6)	<1 (0)	$< 1{,}25 \times 10^{-9}$
J2315 (pEF32)	78 (-4)	1000 (-6)	<1 (0)	$< 1{,}28 \times 10^{-6}$
J2315 ΔPtw (pEF31)	22 (-6)	320 (-6)	<1 (0)	$< 4{,}55 \times 10^{-8}$

*Number of colonies per plate. Parenthesis shows the dilution reported.
** Transconjugants per donor

5.3 Bacterial Competition Assays

B. cenocepacia has a T6SS which complies with the T6SS archetypical function, as a machinery for bacterial killing (Spiewak et al., 2019). In addition, several reports showed that this system contributes to bacterial survival within the eukaryotic host cell (See Introduction, section 1.3.3). In the experiments carried out by Spiewak et al., the role of T6SS is demonstrated through a series of bacterial competition assays (Spiewak et al., 2019). Furthermore, it is shown that this system has little influence on the establishment of infection in three eukaryotic infection models: *Galleria mellonella*, *Caenorhabditis elegans* and *Danio rerio*.

T4SS may also have bacterial killing activity, such as the T4SS of *X. citri* (Sgro et al., 2019a). In fact, Sgro et al have found a homology between *X. citri*, and some Burkholderiales, for instance, *Hydrogenophaga crassostreae*. Our bioinformatic analysis shows a high homology (>74%) of *X. citri* sequence with two central genes of T4SS of *B. cenocepacia* (VirB4 and VirD4) (Fig 5.5).

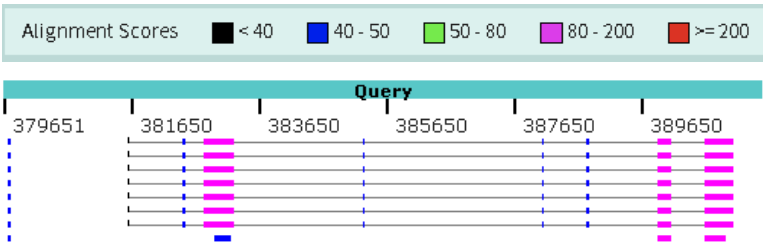

Figure 5.5 BLASTN Top hits between T4SS of *B. cenocepacia* and *X. citri* group. Violet color represents a % identity of approximately 70%. The left region of homology would correspond to *virB4 B. cenocepacia* gene. Second and third (right side) would correspond to *virD4 B. cenocepacia* gene.

Therefore, our objective was to evaluate through the competition methods presented by Spiewak et al., the effect of pT4SS, T4SS, and T6SS, on bacterial competition. The test consists on confronting two bacteria (killer and prey) during a determined killing time (4 hours), and then plating them with specific antibiotics to select each one and calculate the percentage of survival of the prey strain (Figure 5.6).

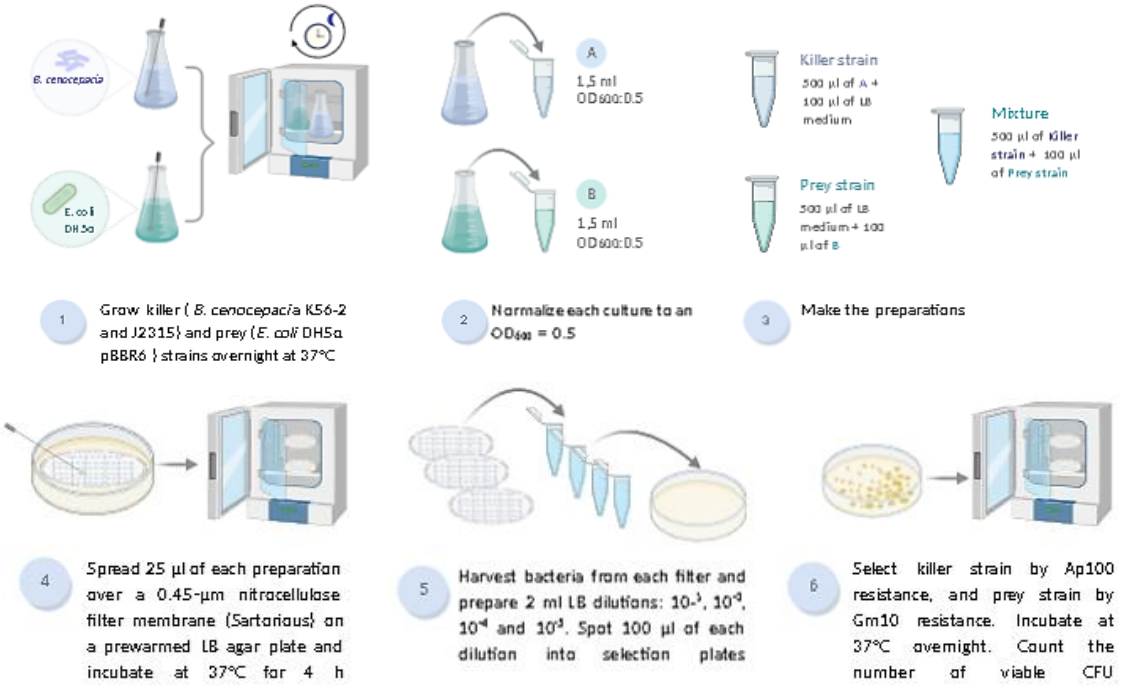

Figure 5.6 Bacterial Competition Assay graphical method.

Different selection methods were tested in order to quantify the killer and prey strains. First, we tested the white/blue phenotype of the colonies on agar plates supplemented with X-gal 40 µg/ml and 0.5 mM IPTG. X-gal is an organic compound, which is oxidized to 5,5'-dibromo-4,4'-dichloro-indigo, a blue insoluble compound. Bacteria that express galactosidase enzyme, coded by *lacZ* gene, change their color to blue. Since *B. cenocepacia* encodes the *lac* operon, we expected it to be blue, so we used as prey an *E. coli* strain, DH5α, which has a deletion in *lacZ* (*lacZΔM15*) and is thus white. In independent experiments, using X-gal and cultivating both strains separately, prey (*E. coli* DH5α) and killer (*B. cenocepacia*), DH5α is observed to acquire a slight blue coloration, whereas *B. cenocepacia* remains being white. This same effect may be observed by cultivating both strains together (Fig 5.7), then adding IPTG, which triggers transcription of the operon *lac*, so the blue color tends to increase. By comparison with the bacterial phenotype previously identified in the separate experiments, it may be established that the blueish strains correspond to *E. coli* DH5α, while the white ones correspond to *B. cenocepacia*.

X-gal IPTG + X-GAL

Figure 5.7 Bacterial competition assay plated on LB supplemented with X-gal (left) or X-gal +IPTG (right). Both images show colonies of *B. cenocepacia* JST39 ΔT4 and *E. coli* DH5α co-cultured in the same petri dish.

The reason why *B. cenocepacia* remains white in the presence of X-gal and IPTG is unknown, but it could be due to low permeability of its envelope to this compound, as reported for other bacteria. As a consequence, the differences in phenotype are not as clear-cut as expected. In addition, co-culture of both bacteria makes difficult to quantitate the prey strain if survival decreases by several logs. Thus, we decided to set up a selection system based on antibiotic resistance. Various plating tests were made in order to check the antibiotic resistance of killer and prey bacteria, as shown in Table 5.4. *E. coli* DH5α is naturally resistant to Nx20. Plasmid pBBR6 was introduced to add resistance to Gm10. *B. cenocepacia* is intrinsically sensitive to Nx20 and resistant to Ap100 and Gm10, but strain K56-2 MHK1 (and its mutant derivatives) is sensitive to Gm10. In summary, the prey strain can be selected against the killer with Nx20 (and/or Gm10 against MHK1 and derivatives), while the killer strains can be selected with Ap100.

Table 5.4 Antibiotic resistance of strains used in bacterial competition assays.

Strain	Role	Ap 100	Gm 10	Nx 20
***E. coli* DH5α (pBBR6)**	Prey	*Sensitive*	Resistant	Resistant
J2315	Killer	Resistant	Resistant	*Sensitive*
J2315 ΔPtw	Killer	Resistant	Resistant	*Sensitive*
K56-2 MHK1	Killer	Resistant	*Sensitive*	*Sensitive*

Three independent competition assays were performed. In all the assays, a negative control of prey-only DH5α was included. The calculation of survival percentage was carried out taking as 100% the growth of this DH5α control. The results are presented in Table 5.5, as survival of

the *E. coli* DH5α prey compared to the survival of the prey strain alone (considered 100%). Fig 5.8 shows the averaged results graphically.

As conclusions from these experiments, we corroborate the involvement of T6 bacterial secretion system in bacterial competition, as reported by Spiewak et al (2019), while showing an interesting new result: the involvement of the T4SS VirB/D4 in bacterial competition. It is important to note that abolishment of the killing activity is observed by deletion of either T4SS or T6SS.

Table 5.5 Bacterial Competition Assay

Killer Strain	Prey Strain	Killer Deletion	% Survival of Prey Strain [1]				
			N° 1	N° 2	N° 3	$\bar{X}$	SD
-	*E. coli* DH5α (pBBR6)	-	100	100	100	100	-
J2315	*E. coli* DH5α (pBBR6)	-	4,0	4,2	0,6	2,9	2,0
J2315ΔPtw	*E. coli* DH5α (pBBR6)	Ptw plasmid	0,3	1,9	26,7	9,6	14,8
K56-2 MHK1	*E. coli* DH5α (pBBR6)	-	1,0	2,8	2,2	2,0	0,9
K56-2 JST17	*E. coli* DH5α (pBBR6)	pT4SS	0,10	0,10	0,01	0,1	0,1
K56-2 JST39	*E. coli* DH5α (pBBR6)	T4SS	20,0	139,2	142,4	100,5	69,8
K56-2 JST52	*E. coli* DH5α (pBBR6)	pT4SS, T6SS	84,0	61,5	129,1	91,5	34,4
K56-2 JST150	*E. coli* DH5α (pBBR6)	T2SS, T3SS, T6SS	80,0	103,1	87,3	90,1	11,8

[1] Data represent the percentage of prey colonies recovered after the killing assay, relative to the number of colonies recovered without the presence of a killer strain (100% survival). Each column shows an independent assay

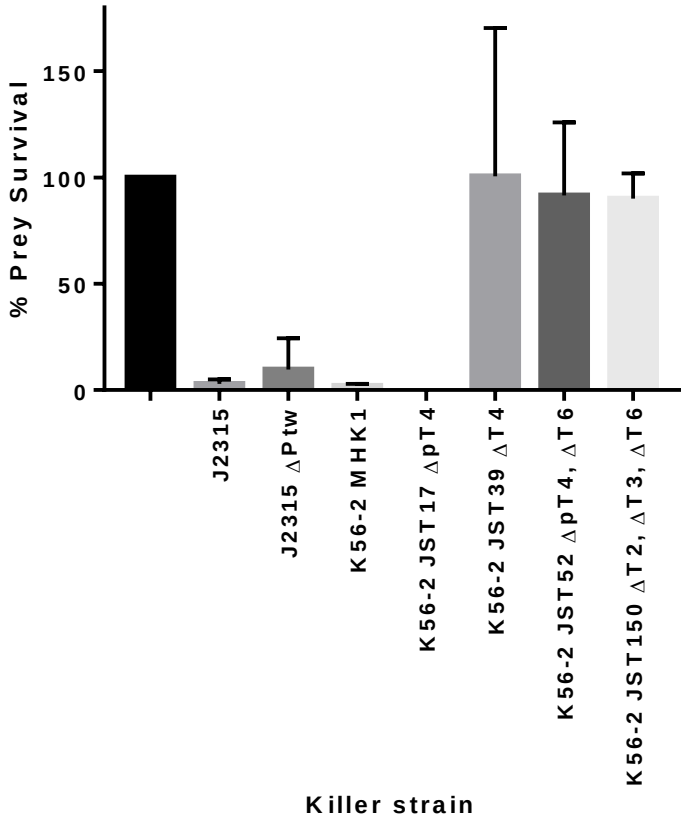

Figure 5.8 Prey Survival in competition assays. Each bar represents the average of % survival of the three independent experiments shown in Table 53. Vertical bars represent the standard deviation. The leftmost bar represents the survival of the prey strain in the absence of killer strain, which was normalized to 100 %.

5.4 Virulence Assays

In order to evaluate the role of *B. cenocepacia* secretion systems in its virulence, two different types of experiments have been reported: the observation of induction of watersoaking in plant tissues upon bacterial infection (Engledow et al., 2004), and the ability of *B. cenocepacia* to survive inside macrophage cells (Sajjan et al., 2008). In addition, we aimed to test DNA transfer to human cells as a new possible role of *B. cenocepacia* T4SS, which could contribute to its virulence.

5.4.1 Plant tissue watersoaking assay

The watersoaking assay consists on the observation of the presence of tissue damage in onion scales upon infection with the bacteria (Engledow et al., 2004). The test consists on inoculating a small amount of bacteria on an onion scale, maintaining aseptic conditions. The onion has an incision on which the bacterial inoculum is deposited. After 24 hours it is possible to observe how the tissue releases a drop of water, which is named as the watersoaking effect.

In order to optimise the assay, a number of factors were analysed:

- The concentration of bacteria: trials were carried out with overnight cultures, in which a greater pathogenicity of the tissue was observed. However, under these conditions, it was not possible to standardize the effects, since the amount of the inoculum was possibly different. Therefore, the assays were performed with cultures adjusted to an OD_{600} of 0.5. The same cultures were plated at the same time to count CFUs and verify their concentration.
- The humidity of the environment surrounding the onion scale had to be controlled by adding water to the onion container, since it was observed that the water drop was not visible when the humidity environment was not appropriate. A moisture evaporation prevention environment was used in the experiments.
- Tests were performed using onions that had not been previously washed with sterile water, in which case a milder watersoaking phenotype was observed, compared to onions that were washed for 30 minutes. This could reflect the existence of antibacterial compounds in the onion surface.
- The watersoaking phenotype is also influenced by the incubation time. At 16 hours it is possible to observe the drop of water, which remains until 24 hours. At longer times, this may be affected due to the evaporation of the liquid, causing significant differences.

Once the optimal conditions for the assay were established, the watersoaking phenotype caused by several wild-type and mutant strains in secretion systems (T2, T3, pT4, T4 and T6) was evaluated. The results are shown in Figure 5.9 (representative assay) and Table 5.6 (three independent assays).

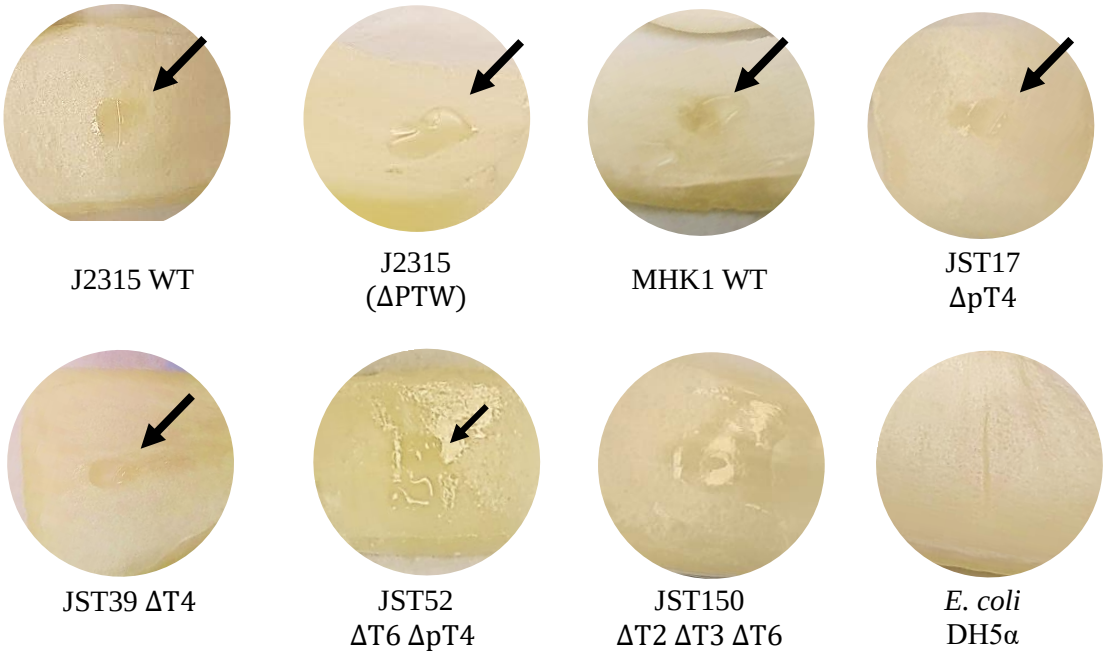

Figure 5.9 Representative photographs of the watersoaking phenotype assay. *B. cenocepacia* wild-type *strains* MKK1 and J2315 and *E.coli* DH5α were used as positive and negative controls, respectively.

Table 5.6 Onion tissue watersoaking assay

Strain	Deletion	Number of assay [2]			AVERAGE
		N° 1	N° 2	N° 3	
J2315	-	++	+++	+	++
J2315ΔPtw	Ptw plasmid	+++	+++	++	+++
K56-2 MHK1	-	++	+++	+++	+++
K56-2 JST17	pT4SS	++	+++	+	++
K56-2 JST39	T4SS	++	+	+++	++
K56-2 JST52	pT4SS, T6SS	++	+	+	+
K56-2 JST150	T2SS, T3SS, T6SS	+	+	++	+
E. coli DH5α	-	-	-	-	-

[1] Secretion system deleted
[2] Each column shows an independent assay

It can be observed that all tested *B. cenocepacia* strains produce some level of watersoaking, compared to the *E. coli* control (Fig. 5.9). In particular, no decrease in the watersoaking phenotype was observed in the pT4SS mutants, both in *B. cenocepacia* K56-2 and J2315. The differences in the size of the water drop was found to be very variable among assays (see Table 5.6), precluding us from taking any further conclusions on the role of any SS in the ptw phenotype. We also qualified the appareance of other tissue damaging indicators, such as blackening, and tissue deformation., but the phenotypes were variable and no consistent results could be observed, so the assessment of these phenotypes was discarded.

It was surprising to observe that the strain J2315 lacking the Ptw plasmid promoted higher levels of watersoaking compared to the wild-type strain, in contrast with what was previously published (Engledow et al 2004). In order to check that these strains were correct, a verification PCR was performed on total DNA to amplify a region of *ptwC*, which should not be present in the strain cured of the Ptw plasmid. The results are shown in Figure 5.10. The expected amplification band is visible in the wild-type strain and absent in the strain cured of the plasmid, as expected. Thus, the main conclusion of this assay is that the pT4SS of *B. cenocepacia* is not responsible for the plant tissue watersoaking phenotype.

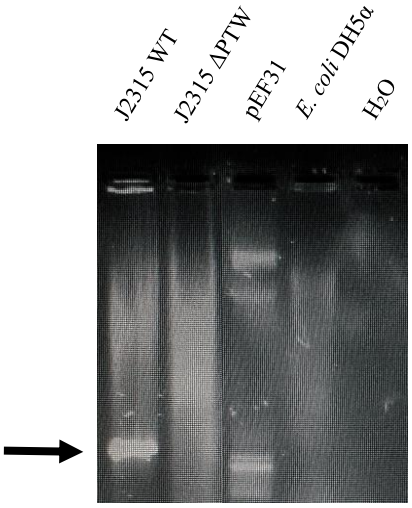

Figure 5.10 PCR analysis of J2315 and ΔPTW, plasmid pEF31 containing the Ptw region, used as positive control, and *E. coli* DH5α used as negative control.

5.4.2 DNA transfer to human cells (planned).

Several previous evidences suggested that pT4SS-mediated DNA transfer could occur from *B. cenocepacia* into its eukaryotic host cell. First, the presence of a relaxase PtwC, and the ability of Ptw plasmid to mediate conjugative DNA mobilization (Fernández-González et al, 2016), proved that the pT4SS can mediate DNA transfer. Second, the expression pattern of the *ptw* genes upon contact with plant components, suggests a role in pathogen-host interaction (E. Fernández-González, 2012, PhD thesis). Thus, the possibility exists that pT4SS could be used to transfer genetic material to the eukaryotic host, as has been observed in other T4SS involved in pathogenicity (Fernández-González et al., 2016; Guzmán-Herrador et al., 2017). In these reports,

DNA transfer to human cells was assayed with the methodology put forward in our lab, based on the transfer of a plasmid encoding a eukaryotic GFP expression cassette. DNA transfer is detected and quantified by the percentage of cells emitting green fluorescence (Fernández-González et al, 2011).

DNA transfer from *B. cenocepacia* to human cells was assayed without success (Fernández-González, 2012), but the experiment had the limitation that infections could not be maintained for a long term since cells died after 24-48h; gentamycin could not be used to kill remaining extracellular bacteria, because *B. cenocepacia* is naturally resistant, and other antibiotics tested did not efficiently remove extracellular bacteria, leading to the premature death of the infected cells (E. Fernández and P. Guridi, unpublished). It is probable that the DNA transfer cannot be detected until 78 hours post infection, as in the case of *B. henselae* T4SS (Fernández-González et al, 2011). The newly acquired gentamycin-sensitive *B. cenocepacia* strains from M. Valvano (see Table 5.5) would allow us to maintaining the infection during more than 72 h. Unfortunately, the lockdown due to the Covid-19 pandemic precluded us from testing these strains for DNA transfer to human cells.

These strains will also allow us to carry infections of IB3 cells and check for intracellular survival of wild-type and mutants. Carrying out these assays could be important due the contradictory reports about the contribution of pT4SS to intracellular survival (see Introduction section 1.3.2). Preliminary assays carried out in our laboratory (Pablo Guridi, unpublished results) using gentamycin sensitive strains confirmed that gentamycin treatment can be applied to kill all extracellular bacteria, thus preserving the viability of the infected cells for longer time. However, the infection rate was only around 0,5%. Future optimization of infections with the gentamycin sensitive strains will shed light into the role of SS in intracellular survival, and will pave the way to assess DNA transfer to eukaryotic cells.

6. Discussion

In the present research work, an approach was made in order to study the biological role of T4SS and T6SS in *B. cenocepacia*, with the aim of establishing their involvement in functions such as DNA transfer, pathogenesis, and bacterial competition.

6.1 Involvement of pT4SS in conjugative DNA mobilization

In in order to confirm the involvement of the pT4SS in conjugative DNA transfer, as a first step we aimed to reproduce previous results about the mobilization of the plasmid pEF31 by the *B. cenocepacia* Ptw conjugation functions (Fernández-González et al, 2016), to subsequently assay the pT4SS mutant strains. However, after several replicates where different conditions were evaluated, such as the preparation of antibiotics and their concentrations, the addition of onion extract, or the resuspension of the conjugation mixture in SOC (conjugation promoter medium), no transconjugants were obtained (Table 5.2 and 5.3). According to E. Fernández-González, these conjugations did not always render transconjugants (Fernández-González, 2012). The conditions that determine this DNA transfer are probably not completely standardized. Thus, a greater number of conjugation assays would be required in order to confirm the previous result. It is important to highlight that in collaboration with other laboratory project, conjugation assays in *E. coli* were executed applying the same method described above and transconjugants were obtained. This rules out possible methodological mistakes. With the present results, we cannot confirm nor reject the previous conclusions on the role of the Ptw plasmid in conjugative DNA mobilization.

The reason why conjugal mobilization is not reproducible is unknown, but we can speculate that this is probably a naturally repressed conjugation system, which will only be expressed under specific environmental conditions, which are hard to reproduce in the laboratory. It must be noted that Fernández-González et al. obtained low frequencies of mobilization, which could be due to the fact that the mobilizable plasmid had multiple copies, thus possibly titrating out a conjugative repressor. Other natural conjugative systems constitutively repressed were only evidenced under laboratory conditions after many efforts (Pérez-Mendoza et al., 2004).

6.2 Role of T4SS in Bacterial Competition

Among the many biological roles of T4SS, bacterial killing was the last to be described (Souza et al., 2015). The T4SS of *X. citri*, which mediates killing of surrounding prey strains, was shown to share some homology with the T4SS of Burkholderiales (Sgro et al., 2019b) (Fig 6.1). In order to assess a possible role of *B. cenocepacia* T4SS in bacterial competition, a bacterial

killing assay was set up, and *B. cenocepacia* strains carrying deletion mutations of different secretion systems (pT4SS, T4SS, T6SS, T3SS and T2SS) were assayed for their ability to kill a prey *E. coli* strain when co-incubated on a solid surface. It has already been reported that T6SS actively participates in bacterial competition (Spiewak et al., 2019). However, until now, the involvement of T4SS from *B. cenocepacia* in bacterial competition has not been documented.

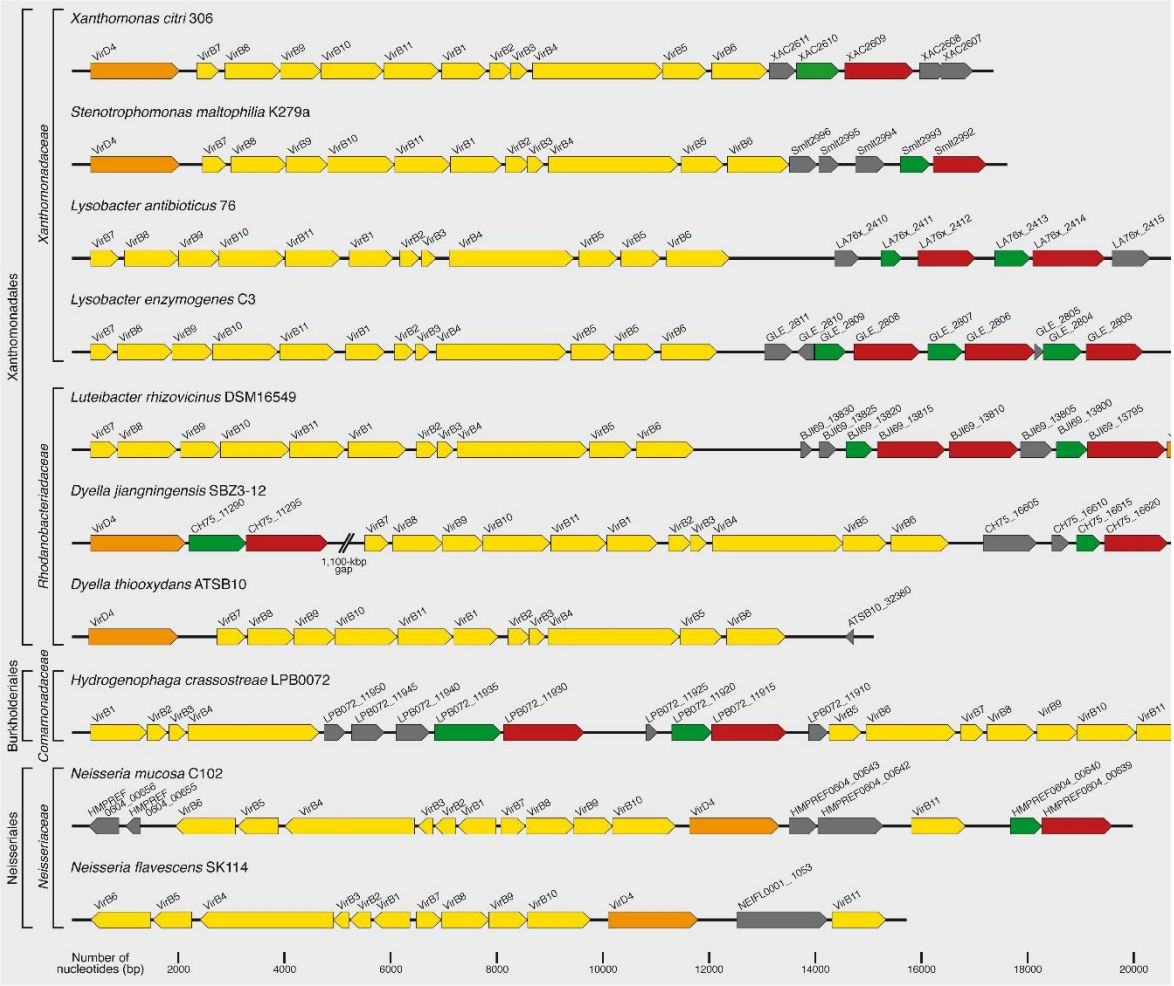

Fig 6.1 *X. citri* chromosomal *vir* locus and its homologs in other species, such as *Hydrogenophaga crassostreae* LPB0072, belonging to Burkholderiales. *virB* and *virD4* genes are shown in yellow and orange, respectively. Xanthomonadales-like T4SS effectors (X-Tfes) and immunity proteins (X-Tfis) are colored red and green, respectively. Other open reading frames coding for proteins of unknown function are shown in gray. Taken from (Sgro et al., 2019b).

The results of the bacterial killing assays (Table 5.5 / Fig 5.8) corroborate that the T6SS has bacterial killing activity, since strains with mutations in the T6SS suffered a notable decrease in their ability to kill the prey strain. Concerning T4SS, deletion of pT4SS does not affect the killing ability of *B. cenocepacia*. However, most interestingly, an increase in the survival percentage of the prey strain is observed when it is faced with ΔT4SS *B. cenocepacia* strains, in comparison

with wild type strain (Figure 5.8). It is important to mention that a similar affectation in the killing capacity is observed both in the strains lacking T6SS (JST52, JST150), and in the strain lacking T4SS (JST39). Although JST39 possesses the genes that code for T6SS, it appears to be non-functional in this T4 mutant strain. If both T6SS and T4SS were independent bacterial killing systems, we would expect a certain killing capacity to be present in each mutant, and only the double mutant would have abolished this capacity. However, either mutation has a similar effect. Therefore, it appears that the T6SS in *B. cenocepacia* loses functionality when the T4SS is affected. This would suggest that both systems act in coordination. T6SS have a versatile way of acting, either as a proper attack mechanism, as is the case of *Vibrio cholerae*, or a defense mechanism, which only responds when it detects the attack by another T6SS, as is the case of *Pseudomonas aeruginosa* (Basler et al., 2013). That means that a complex regulation network may be required to trigger T6SS action.

Our results suggest that somehow the T4SS is controlling the expression of the T6SS. Different scenarios have been described for such regulatory interaction between T4SS and T6SS, many involving plasmid-encoded elements which control the regulation of a co-resident T6SS (Peñil-Celis & Garcillán-Barcia, 2019). Among those, cell-cell interactions mediated by the T4SS of plasmid RP4 in *E. coli* trigger H1-T6SS counterattack of *P. aeruginosa*, resulting in a decreased survival of the donor *E. col* (Peñil-Celis & Garcillán-Barcia, 2019) (Fig 6.2d). In other words, T4SS components seem to trigger a T6SS response, a situation which could be similar to what we observe, with the difference that in this case, a chromosomally encoded T4SS regulates the T6SS present in the same genome.

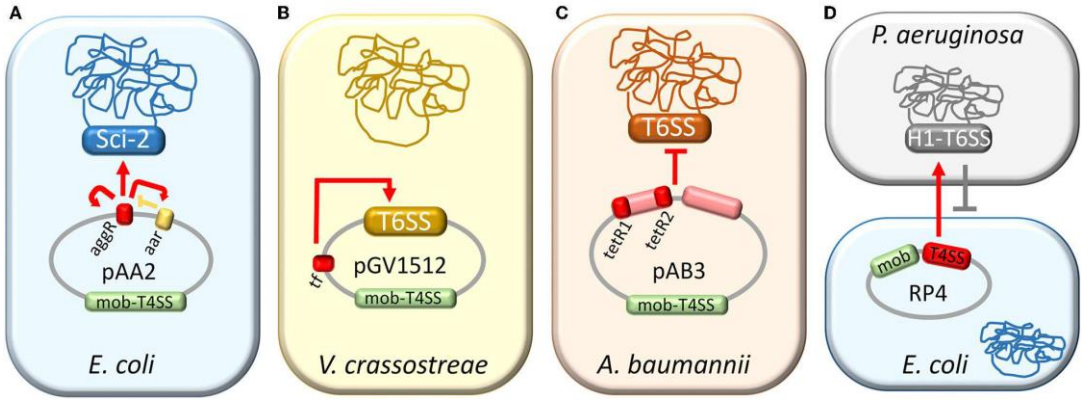

Fig 6.2 Control of T6SS activity by plasmids. Plasmid-mediated regulation of T6SSs in *E. coli* (A), *V. crassostreae* (B), *A. baumannii* (C), and *P. aeruginosa* (D). Plasmid regions involved in T6SS regulation are depicted in red. (A) pAA2-encoded auto-regulator AggR activates the Sci-2 T6SS (red arrows) and is negatively regulated by Aar (yellow lines). (B) Transcriptional factor (TF) of pGV1512 activates the plasmid-encoded T6SS (red arrow). (C) Two regions of the pAB3 plasmid, one of them including tetR-like regulators, repress the T6SS of *A. baumannii* (red lines) (D) Cell-cell interactions mediated by the T4SS of plasmid RP4 trigger H1-T6SS counterattack (red arrow), resulting in a decreased survival specifically in plasmid-bearing cells (gray lines). Taken from (Peñil-Celis & Garcillán-Barcia, 2019)

In summary, the VirB T4SS of *B. cenocepacia* appears to be involved in bacterial killing in coordination with T6SS. Previous work shows a T4SS role in bacterial killing in *X. citri*. However, in *X. citri* it is the T4SS itself which performs the antibacterial activity. Furthermore, effector-immunity protein pairs were described associated with this T4SS, suggesting a similar toxicity mechanism as for T6SS. (Sgro et al., 2019b). Our bioinformatic search shows high homology (approximately 80%) only for VirB4 and VirD4 proteins, while it is very low for the rest of the system (Figure 5.5). This suggests that the VirB T4SS may not be a bacterial killing machine. Taking in consideration that a role for bacterial killing has been proven for the T6SS of *B. cenocepacia*(Spiewak et al., 2019), and that killing is abolished by the mutation of either SS (T4 or T6), we favor the hypothesis that T6SS is the actual killing machine, while the presence of the T4SS is also required for this phenotype, for instance by driving the expression of the T6SS genes.

We planned to continue this investigation by conducting a study of gene expression profiles of T4 and T6SS genes, using RT-PCR, in order to check whether both systems are expressing themselves during bacterial killing, and if the expression of one set of genes affects the expression of the other. If we verify that these groups of genes are being expressed at significant levels during bacterial competition tests, and not under standard growth conditions, we could suggest that both systems are related to this phenotype. Moreover, we could check if the expression of the T6SS genes is affected by the absence of the T4SS. Our hypothesis is that the T4SS mutant strain will show lower or null expression of T6SS genes compared to the wild type. If the gene expression profiles for both systems do not show significant changes, it would still be possible that our method of differentiation is not sensitive enough to quantify this expression, or that the interaction of these systems is established at the protein level, therefore, antibody-mediated inmunodetection assays could be performed in the future.

6.3 Assessment of T4SS role in bacterial virulence

Several reports have suggested a role of the pT4SS in *B. cenocepacia* interaction with the eukaryotic host cell: it was reported that a mutant in pT4SS genes abolished the plant tissue watersoaking phenotype (Engledow et al., 2004), and that pT4SS contributed to *B. cenocepacia* survival inside macrophage cells (Sajjan et al., 2008). As detailed in the Introduction (Section 1.3), some other reports shed contradictory results. Thus, we decided to test our set of wild-type and mutant strains in both assays. In addition, since our previous results related the pT4SS to DNA transfer, the pT4SS genes are expressed upon contact with the eukaryotic cell, and our group has reported the existence of T4SS-mediated DNA transfer to eukaryotic hosts (Fernández-

González et al, 2011), we also aimed to test this phenotype in *B. cenocepacia* wild-type and mutant strains. The laboratory lockdown has prevented us from completing this section, but still the expected outcomes are discussed here.

6.3.1 pT4SS is not involved in Plant Tissue Watersoaking

We have reproduced the plant tissue watersoaking assay as is originally described by (Engledow et al., 2004). This assay consists in observing the presence of a water droplet on the onion surface when it is inoculated with *B. cenocepacia*. We found that the presence and size of the droplet may be influenced by several aspects, such as the amount of bacteria inoculated, the humidity of the environment, or the antibacterial potential of the onion tissue, when it is not washed with sterile water. We established the optimal conditions in order to obtain the watersoaking phenotype, that includes infection with a fixed amount of bacteria, washing onion scales during 30 min, providing a humidity controlled environment, and observing the phenotype at 24 hours post infection.

We performed the assay with the set of wild-type and mutant *B. cenocepacia* strains. The most interesting conclusion that can be drawn from these assays is that the watersoaking phenotype is independent of the pT4SS genes, as well as any of the genes encoded in the plasmid named Ptw, since the strain J2315 ΔPtw lacks the whole functional plasmid and produces watersoaking (Figure 5.9). This result contradicts what was previously reported (Engledow et al, 2004). This test was performed in triplicate, obtaining similar results, and in all assays, the positive and negative controls were correct (Fig 5.9 and Table 5.6). In addition, we confirmed the absence of the *ptw* genes in the ΔPtw strain by PCR (Fig. 5.10). The reason why other authors obtained a watersoaking deficient *ptw* mutant are unknown, but it could be related to the many factors affecting its outcome. A false negative is easy to explain in this assay, while the probability that our result is a false positive is remote.

Our results unlink the presence/absence of *ptw* genes with the watersoaking phenotype. This may explain why the conjugative DNA transfer performed by Fernández-González et al (2016) worked equally well to two *B. cepacia* recipient strains, in spite of their different watersoaking phenotype. In fact, as recipient strain to test for Ptw-mediated conjugative DNA transfer, the authors looked for a strain that was similar enough to *B. cenocepacia* but did not present *ptw* genes, to avoid the phenomenon of plasmidic DNA entry exclusion: recipients carrying a plasmid prevent incoming conjugation of that same plasmid (Garcillán -Barcia & de la Cruz, 2008). In the absence of the genomic sequence of these strains, the presence/absence of *ptw* genes was inferred by their watersoaking phenotype. Figure 6.1 shows the difference in watersoaking phenotype in both recipient strains, compared with a negative control of *E. coli*

DH5α. At that time, it was thus surprising to see that both strains behaved equally well as recipients of Ptw-mediated conjugation. However, in view of our results, the genes encoding pT4SS and the watersoaking phenotype do not appear to be related to each other, thus explaining the results obtained by Fernández et al. where transconjugants are observed in both recipient strains.

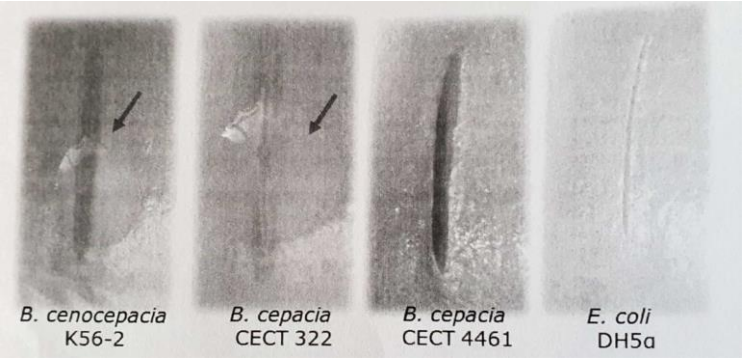

Fig. 6.1 Watersoaking phenotype in *Burkholderia cepacia*. *B. cenocepacia* K56-2 and *E. coli* DH5α were used as positive and negative controls respectively. Taken from Fernández-González, 2012.

Our results further show that none of the mutant strains abolished the watersoaking phenotype (Fig. 5.9), implying that no secretion system is responsible on its own for the phenotype. Probably, several mechanisms may be acting coordinately to cause tissue damage. Apart from this fact, we consider that no further conclusions can be drawn from the size of the droplet, due to the high variability of the Ptw assay, unless many more assays were performed. We also tried to quantify other tissue damaging aspects, but discerning the phenotypes was neither easier nor more informative than the watersoaking effect. This study was carried out as an initial screening. In case we want to go deeper into this study, we would look for quantitative methods to assess pathogenicity, for example, quantifying protein expression levels of selected genes during the infection process.

6.3.2 Human Cell Infections and DNA transfer to human cells (expected outcomes)

Previous attempts carried out in our laboratory in order to set up the *B. cenocepacia* cell infection assays were challenging. This was mainly because the infections could not be maintained for more than 24 hours, since the cells died. The infection test requires that the bacteria are in contact with the eukaryotic cells for 2 hours, after which, the bacteria that have not entered the eukaryotic cell must be washed and eliminated with antibiotics, so that they do not compromise the cell viability by continuous infections. With *B. cenocepacia* wild type strains,

the process of washing and eliminating extracellular bacteria was inefficient because the antibiotics used were not entirely effective in selecting them. By using the strains obtained from M. Valvano, which are sensitive to gentamycin, this problem could be overcome. Due to the laboratory lockdown, we could not perform these infections and test the effect of the pT4SS deletion on intracellular survival, on which there are contradictory reports (Valvano, 2015) (Sajjan et al., 2008). Very preliminary assays from short-term infections (Pablo Guridi, unpublished) show little, if any, effect of the pT4SS deletion on intracellular survival. Our working hypothesis is that pT4SS absence won't affect intracellular survival, in line with our previous result on the absence of effect of pT4SS in the watersoaking phenotype, strengthening the concept that pT4SS is not involved in virulence.

The lockdown also precluded us from testing whether pT4SS plays a role in DNA transfer to human cells, as demonstrated for other T4SS from the human intracellular pathogens *B. henselae*, *Legionella pneumophila* or *Coxiella burnetii* (Fernández-González et al, 2011; Guzmán-Herrador et al, 2017). A sustained infection model is required over time for DNA transfer to be detected. Again, the current availability of *B. cenocepacia* Gm-sensitive strains should allow the establishment of such long-term infections, so that we could check the DNA transfer from *B. cenocepacia* to human cells. The experimental setup is established and the appropriate constructs to detect transfer by GFP expression in the human cell are already constructed. A positive result would be very significant, and the following step would be to check the mutant strains. Our hypothesis is that, if there is DNA transfer from *B. cenocepacia* to human cells, this would be mediated by the pT4SS, which encodes the DNA relaxase PtwC, required to lead the DNA through the T4SS.

7. Significance, further research and applications

This work presents an approach to the study of the biological role of the T4SS of *B. cenocepacia* and collects relevant information about the findings and contradictions reported for its various secretion systems. *B. cenocepacia*, an opportunistic pathogen, has been shown to have complex and versatile biological machinery, both for pathogenicity and DNA transfer. The study of the roles in which each of the mechanisms are involved has a long way to go. However, basic information has been contrasted to guide future research, regarding the study of secretion systems in *B. cenocepacia*.

In this work, important limitations for *B. cenocepacia* cell infections and DNA transfer assays have been highlighted, and we have paved the way to solve them in the near future. The significance of a possible transfer of genetic information from the pathogen to the human host indeed justifies to pursue this line of research. If confirmed, it will be of outmost interest to determine which information is transferred, and with which purpose.

A possible new role not previously described for T4SS in *B. cenocepacia* has been established, which is to participate actively and in conjunction with T6SS, to produce bacterial killing. Apart from the novelty of this finding, it must be stressed that bacterial competition may be relevant in a clinical context, considering that *B. cenocepacia* must thrive in the lung of CF patients, where a complex microbiota is developed.

Finally, our results conclusively prove that pT4SS is not involved in the plant tissue watersoaking phenotype, in contrast with previous reports. This pT4SS is likely devoted to DNA transfer among bacteria, and it is still to be tested if also to the eukaryotic host. Our bioinformatics analysis shows that this system was laterally acquired and is not widespread amongst the Bcc, while the T4SS or T6SS are highly conserved. Thus, it is more likely that the latter play a role in virulence, while the pT4SS may play an accessory role contributing to *B. cenocepacia* adaptability.

These findings represent an interesting insight into the roles of secretion systems in the biology of a human pathogen, and initiate an additional approach to the versatility of secretion systems in *B. cenocepacia*. Understanding how these systems work together can give us a more detailed understanding of the virulence mechanisms of this opportunistic pathogen for both plants and animals. In the long term, this line of research may give us important clues for establishing effective pharmacological targets for its treatment, or even in basic research, giving insight into the functioning of secretion systems in the complex interactions between pathogens and their environment.

8 Conclusions

1. The *B. cenocepacia* VirB T4SS and T6SS are highly conserved among the *B. cepacia* complex, while the Ptw pT4SS is poorly conserved, suggesting a recent acquisition by horizontal transfer.

2. We have not been able to reproduce the conjugative DNA mobilization of plasmids between *B. cenocepacia* and *B. cepacia* CECT322.

3. *B. cenocepacia* mutant strains lacking T2, T3, T4, or T6 secretion systems maintained the capacity to produce the plant tissue watersoaking phenotype.

4. *B. cenocepacia* K56-2 ΔpT4SS and J2315 ΔPtw mutants did not differ from their respective wild type strains in plant tissue watersoaking phenotype. Thus, the pT4SS is not responsible for this phenotype.

5. In a bacterial killing assay against *E. coli* (prey strain), an increase in prey survival is observed when the killer strain is *B. cenocepacia* ΔT4SS compared to the wild type strain.

6. *B. cenocepacia* mutants in either VirB T4SS or T6SS lose their bacterial killing capacity, suggesting that both secretion systems are implicated in the same bacterial competition process.

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